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13. ABSTRACT (Maximum 200 Words)

In the present report we describe studies using chimeric immune receptors consisting of an antibody fragment against the CEA tumor antigen joined to signaling portions of the T cell receptor(IgTCR). The antibody fragment confers CEA specificity to the receptor, while the signaling domains transmit T cell activation signals. These receptors allow the T cells (designer T cells) to bypass immune tolerance to CEA and to activate effector functions in a tumor specific manner. We have partially completed a phase I study using IgTCR-modified designer T cells. These studies demonstrate the specific signaling molecules activate specific T cell effector functions in a tumor specific manner. The study will be completed on a continuing no-cost extension with the funds allocated.

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Table of Contents

4. INTRODUCTION

Carcinoembryonic antigen (CEA) is a tumor-associated antigen which is expressed on ≈30-60% of metastatic breast tumors. The purpose of this research is to develop a new type of cancer therapy using autologous T cells modified with a chimeric immunoglobiin T cell receptor (IgTCR) directed against CEA + tumors. The specific objectives are to:
1. Complete setup for therapy.
2. Apply IgTCR-modified cells in a phase I clinical study in patients with metastatic CEA + breast tumors

In addition, to the above we have also carried out basic research efforts to create second-generation reagents that will enhance the therapy for future phase II/III studies.

5. BODY

The following activities were undertaken under the current year no-cost extension to the above referenced grant. It is noted that only two years of funding were provided by the DOD, whereas the task list was stated for the entire 4 year requested funding period. Accordingly, certain of the tasks were abandoned due to lack of funding, as indicated below.

I. Lab and clinical set up for therapy.

This was previously reported as completed.

However, we have been mandated to redo this step during the recent year by our local institution (not by the FDA), with a patient-dedicated facility. This was previously in a shared-use facility according that was acceptable under FDA specifications. We have hired new staff and are presently re-establishing this facility. We anticipate certification for resumption of patient treatments in 9/2001.

II. Phase I clinical trial

This was trial was partially completed but interrupted due to reasons mentioned in the prior report. In the past year, we have responded to requests for redesign of the Cell Transduction Facility (CTF) for patient sample preparation. We have also established a DSMB to conduct patient monitoring on this gene therapy study. With various other administrative responses and design changes (only very modest changes to the treatment protocol), we are ready to resume the study in 9/2001 and should complete the trial within the coming year. There remain 5 patients to treat on the phase I study.

All patient care funds have been preserved, and will be applied to complete the phase I tests.

III. Phase II study

Funding was not provided to support this study. This task is abandoned.

IV. Phase II study +IL2

Funding was not provided to support this study. This task is abandoned.

V. Laboratory studies for therapy improvement.

A. Prepare improved VPCs.

Completed in prior report.

B. Prepare improved helper cell lines.

Funding was not provided to support this effort. This task is abandoned.

C. Prepare CEA-IgG chimeric protein.

This product has been designed and cloned. It has yet to be expressed, which will be conducted during the next year of this no-cost extension.

6. KEY RESEARCH ACCOMPLISHMENTS
- Re-certify newly patient dedicated Cell Transduction Facility.
- New staff hiring and training.
- Established DSMB.
- Patient Treatment objectives deferred. To be accomplished with remaining funds.
- Preparation of CEA-IgG construct.

7. REPORTABLE OUTCOMES
- Publications:

    Beecham EJ, Ma QZ, Ripley R, Junghans RP. Coupling of CD28 co-stimulation to IgTCR molecules: Dynamics of T cell proliferation and death. J Immunother 2000;23:631-42.

    Ma QZ, Gonzalo-Danganzo R, Junghans RP. Genetically engineered T cells as adoptive immunotherapy of cancer. In Harrison P (ed) Cancer Chemotherapy & Biological Response Modifiers – Annual 20 Oxford: Elsevier Science. 2001: in press.

8. CONCLUSIONS

The key clinical efforts of this study are deferred and will be completed in the coming year with conserved Department of Defense funds under this no-cost extension.

9. REFERENCES

None.

10. APPENDICES

A. Manuscripts

Beecham EJ, Ma QZ, Ripley R, Junghans RP. Coupling of CD28 co-stimulation to IgTCR molecules: Dynamics of T cell proliferation and death. J Immunother 2000;23:631-42.

Ma QZ, Gonzalo-Danganzo R, Junghans RP. Genetically engineered T cells as adoptive immunotherapy of cancer. In Harrison P (ed) Cancer Chemotherapy & Biological Response Modifiers – Annual 20 Oxford: Elsevier Science. 2001: in press.

Genetically Engineered T Cells as Adoptive Immunotherapy of Cancer

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1. Introduction

1.1. Immunotherapy

It has been a major focus of modern cancer research to explore the potential of immune system components to combat malignant disease. This strategy has been two-pronged, exploiting on the one hand the humoral immune system as represented by antibodies, and on the other the cellular immune system as represented by lymphokine activated killer cells and tumor infiltrating lymphocytes. In general, antibodies have the capability to supply specificity, but suffer from lack of potency. Cellular therapies, in contrast, have potency with potentially lethal consequences if not closely regulated, whereas specificity is often lacking.

1.2. Adoptive immunotherapy

Evidence from experimental animal tumor models and cancer patients showed that cellular rather than humoral immune responses were responsible for the rejection of tumor [1, 2]. Effective cellular immune therapy in cancer needs three essential components:

1. Tumor recognition (specificity)
2. Cytotoxicity against tumor cells (potency)
3. Sustained activity in the tumor bed (effector cell survival/proliferation)

These components will be referred to throughout this essay. To address these features, alternative approaches explored in recent years have been to genetically engineer T cells to express cytokines or chimeric receptors for tumor antigens to make the T cells either more potent or more specific. By way of background, a brief history of the use of LAK

cells and TILs will be presented while the remainder of this chapter will focus on the genetic manipulation of T cells.

1.2.1. LAK cells

Early studies with adoptive immunotherapy applied lymphokine activated killer (LAK) cells derived by ex vivo amplification of autologous lymphocytes with interleukin-2 (IL-2) [3]. Large numbers of cells were infused, as many as $4x10^{11}$, in a regimen with co-administration of IL-2. Substantial numbers of responses were obtained in renal cell carcinoma and melanoma (21-35% CR+PR), but the added benefit of LAK + IL-2 versus IL-2-alone, which also generated responses, was marginal [4]. Substantial toxicities were generated, but these toxicities did not appear to differ from those of the IL-2 alone. The LAK treatments were applied at maximum tolerated or maximum practical doses, yet a conclusive benefit of the LAK cell treatments could not be shown independent of the co-administered IL-2.

Whereas the LAK cells were shown to demonstrate cytotoxicity against tumor cells in vitro against cell lines [5], and in animal models using such cell lines [6], their in vivo application in humans lacked sufficient specificity and potency. However, the viability of such cells was successfully sustained by IL-2 administration, which causes LAK cell expansion in vivo [7].

1.2.2. TILs

When responses were observed with IL-2 therapy alone, this was correlated with T cell infiltration and proliferation in the tumor bed. It was hypothesized that these T

3

infiltrating lymphocytes (TILs) were tumor-specific, and part of a natural immune recognition that did not by itself translate into tumor eradication. With the IL-2 stimulus, this native recognition was expanded and efficacy, in a few cases, was achieved. The tumor specificity of the responding T cells was supported by diverse studies, leading to discovery of a large number of new antigens, mainly in melanoma [8]. Curiously, these antigens are typically normal, unmutated proteins derived from normal melanocytes; the basis for their immunogenic character in melanoma is controversial and still under study.

The next generation of adoptive immunotherapies after LAK cells was based on isolating these TILs from tumor specimens, principally in melanoma, and expanding those cells ex vivo, by stimulating the cells with autologous tumor and IL-2. In animal studies, TILs were shown to be 10-100 times more potent than LAK cells in reducing lung metastases [9]. Human trials in melanoma showed TILs trafficking to tumor sites after injection [10-13]. Although the absolute numbers of T cells trafficking to tumor were small, response rates in the range of 30-35% were obtained [14], which were superior to the rates of 20% previously achieved in melanoma with IL-2 alone or LAK + IL-2 [4]. However, the infrequency of durable remissions (<5%) was inadequate to justify the costs and toxicities of therapy.

In patients with cures or major responses with TILs or IL-2 therapy, the three elements of specificity, potency and proliferation were evidently present. Correlates of response in the TILs studies were (1) relative potency of TILs by in vitro cytotoxicity tests, (2) larger numbers of cells infused, and (3) rapid expansion rate of TILs in culture. Whereas cytotoxicity was demonstrated ex vivo by TILs for most patients, this potency was nevertheless inadequate for the task of eliminating tumor masses in vivo in the majority of cases. Examining these three components, the specificity of the TILs was apparent from the fact that the ex vivo T cell expansion was dependent upon the presence of

patient tumor. The potency of patient TILs varied, and this appeared to be a key factor. Finally, the predictive value of the proliferation rate of the cells prior to infusion suggested that the viability and expansion within the tumor was essential also, and insufficient for most patients.

1.3. Need for new approaches

Recognition by T cells may have diverse outcomes, depending upon a number of variables, some of which are still subject to definition. One factor in recognition is the avidity and number of interactions of the TCR for antigen [15, 16]. Where a TCR poorly recognizes antigen, or the number of presented antigen molecules (peptides in MHC) is low, the likelihood that recognition will lead to effective signal generation (for cytotoxicity, cytokine secretion or proliferation) is reduced. Furthermore, it is only the rare setting (e.g., melanoma) in which there is a tumor antigen that generates immune recognition that is not already fully tolerized in vivo. Accordingly, strategies have evolved in order to overcome these deficiencies of the earlier efforts in adoptive immunotherapy, by genetic manipulations that alter the response of patient effector cells to tumor, by increasing potency, specificity and/or proliferation. These are discussed in this chapter.

2. Genetic Manipulation of T Cells

2.1. Transduction Methods

The efficient gene transfer into effector cells is the prerequisite for the application of genetic engineered T cells strategy for adoptive immunotherapy. Numerous techniques are available for gene transfer into mammalian cells [17]. Among them, retroviral based gene transfer is the most effective means presently available for transduction of T cells with high frequencies and stable gene expression.

5

Retroviruses are RNA viruses that are capable of stably integrating DNA within the host genome. Retroviral vectors for gene transfer have been constructed by substituting the gene of interest in place of the viral protein coding regions. These vectors are packaged into retroviral particles using packaging cell lines (Figure 1) [18]. The envelope protein determines the host range of the retroviruses.

The development of packaging cell lines that use alternative envelope genes has resulted in significant advances in the ability to transduce lymphocytes. Specifically, retroviral vectors packaged with the envelope from the gibbon ape leukemia virus (GALV) are capable of transducing T cells with significantly higher efficiencies [19, 20]. Retroviruses require cell division for integrating into the host genome. Typically T cells are activated to proliferate by anti-CD3 antibody. Optimal T cell activation by anti-CD3 plus anti-CD28 can significantly enhance the gene transfer efficiency into human T cells [21]. The presence of fibronectin derived peptide during transduction [22] and application of 1 hour of centrifugation at 1000g can also significantly improve the gene transfer efficiency [19, 23]. By optimized conditions, the transduction efficiency can reach as high as 35-70% of T cells in routine experiments [24, 25].

Recently, T cells modification and expansion by direct DNA transfer has also been shown on a clinical scale [26]. Methods for efficient gene transfer are likely to evolve significantly over the next few years.

2.2. Clinical studies with gene-marked T cells

Before testing clinically the T cells that have been genetically modified with functional genes, Rosenberg and colleagues assayed the safety and viability of T cells modified with a marking gene by retroviral vector in humans [27, 28]. In these initial studies, a retroviral vector (LNL6) derived from the Moloney murine leukemia with the replication

genes deleted, was constructed to express the gene of the bacterial enzyme neomycin phosphotransferase (Neo[R]) (Figure 2A) [29]. This enzyme inactivates G418, a drug toxic to eukaryotic cells that can be used for ex vivo selection. Melanoma-derived TILs were transduced with LNL6 retroviral vector and selected in G418 and expanded. Ten patients were treated with their own autologous gene-marked T cells. Safety assays were performed on the viral supernatant of transduced TILs and on the sera from patients to rule out the existence of replication-competent virus or the malignant transformation of the modified TILs [29]. These modified TILs were traced by polymerase chain reaction in peripheral blood of patients for the first 21 days after infusion and up to 64 days in tumor deposits [27]. Other gene-marking clinical trials were performed by Favrot and Philip [30], Riddell et al [31] and Lotze et al [32], all supporting the safety and lack of toxicity of retroviral gene transduction for human cells.

A primary rationale for the use of TILs has been to exploit the natural homing properties of these immune cells to traffic to tumor deposits [10]. Human trails in melanoma showed TILs trafficking to tumor sites with threefold increase related to non-specific lymphocyte [10-12]. However, Economou et al in a double gene-marking TILs human trial, using marked TILs and PBLs reported no evidence of preferential trafficking of TILs versus PBLs to tumors [13]. In this study, TILs and PBLs from nine patients were activated, expanded in parallel and transduced with different retroviral vectors (LNL6 and G1Na respectively) expressing the Neo[R] gene. Although some patients did experience dramatic antitumor responses, the modified TILs and PBLs were detected in both tumor and normal tissue biopsies without any evidence for preferential TIL trafficking to tumor.

3. Engineering T Cells with Cytokine Genes

Adoptive immunotherapy with LAK cells and later with TILs has shown the need for co-administration of exogenous IL-2 in order to achieve a clinically effective response. While this cytokine is responsible and indispensable for sustaining the proliferation and survival of the effector cells in vivo, the toxicity in humans is high. Efforts have been made to improve by genetic modification these immune effector cells to fight against cancer. Some of these initiatives have focused on making T cells independent of exogenous IL-2, enhancing its sensitivity to IL-2, as well as increasing the antitumor effect by super-expression of tumor necrosis factor (TNF) by such effectors.

3.1. Pre-clinical studies

First described in the late 1980's, the insertion and constitutive expression of the IL-2 gene in a murine T cell line was shown to enable them to grow in an autocrine fashion [33, 34]. Moreover, retroviral expression of the IL-2 gene in human T cell clones showed that these cells retained their antigen specificity and expanded in an IL-2-independent fashion [35]. These investigators also reported a T cell line expressing a chimeric receptor consisting of the extracellular domain (ECD) of the erythropoietin (Epo) receptor and the intracellular signaling domains of the β and γ subunits of IL-2 receptor [36]. These T cells became Epo responsive, demonstrating a functional activation of the IL-2 signal pathway though this chimeric receptor.

Furthermore, genetically modified human CD8+ CTLs expressing a chimeric gene of GM-CSF and IL-2 receptors were shown upon antigen binding to secrete sufficient GM-CSF to support proliferation via an autocrine growth loop in the absence of IL-2, whereas normal CTLs have no receptor for GM-CSF and normally are absolutely dependent on

IL-2 for growth and viability. By this design, modified CD8+ CTLs would have a helper as well as a cytolytic activity, eliminating the necessity of an additional T cell population. This promising approach has been tested so far only in vitro [37].

3.2. Clinical Studies

TNF has shown efficacy as an anti-tumor cytokine capable of inducing regression of experimental murine tumors if adequate levels are achieved [38, 39]. However, the systemic dose required to obtain the desired anti-tumor effect is too toxic in humans. One approach to circumvent this problem was the engineering of TNF-transduced TILs to increase their potency with local cytokine secretion in the tumor. The LNL6 retroviral vector was tested by Hwu et al in a phase I clinical trial without safety or toxicity problems [40, 41]. The vector contained both the human TNF-α gene under the control of LTR retroviral promoter and the Neo^R gene under the SV40 early region promoter. Nevertheless, the level of TNF secretion was almost 30-fold lower than expected when compared to a tumor cell line transduced with the same vector.

In an attempt to improve TNF production, a new version of the vector was engineered by replacing the transmembrane region of the TNF with the signal peptide from IFN-γ (Figure 2B) [29], that increased five-fold the production of TNF in transduced TILs compared to the original vector [42]. This construct was applied in a phase I clinical trial. Although responses were reported, response rates were not notably different versus unmodified TILs alone [40].

Engineering TILs with cytokine genes were among the first attempts to apply gene therapy techniques. Several human studies have reported that retroviral transduction of these immune effector cells with marking and/or cytokine genes is a safe and non-toxic means of therapy for human use. Despite the potential advantages, the expression of

foreign genes in lymphocytes for active cytotoxic secretion is still low and in need further improvement. Other features that could improve the success of adoptive immunotherapy are the enhancement of the migration properties to tumor and the augmentation of tumor specificity and targeting as described in the following section.

4. Engineering T Cells with Tumor Specificity by Chimeric Receptors ("Designer T Cells")

Effective anti-tumor immunity usually cannot be induced due to lack of immunogenicity of the tumor cells. Thus, augmentation of immune effector functions by the infusion of tumor-reactive T cells represents a potentially highly specific modality for the treatment of cancer [43].

T cells conduct their immune functions through their antigen specific receptor, TCR-CD3 complex (Figure 3, right) [44]. The TCR exists in two types of heterodimers composed of two polypeptide chains: α-β (or, in some cases, γ-δ). Each chain of the α-β TCR is composed of a variable region (V) and a constant region (C). The variable region determines the antigen binding specificity of the TCR. The TCR heterodimer is associated with the CD3 complex, which is composed of a group of polypeptide chains: two heterodimers of γ-ε and δ-ε and one homodimer of ζ-ζ. The CD3 complex is important for both the functional expression of TCR on the T cell surface and also for the transduction of the specific signal that is initiated from the TCR.

Upon ligation with the antigenic peptides presented by major histocompatibility complex (MHC) on their target cells, the T cells can be activated to result in cell proliferation, cytokine secretion and cytotoxicity [45, 46].

The antigenic peptides presented by the MHC on the tumor surface and the specific T cells that can react with a particular antigenic peptide are technically difficult to identify and isolate [47]. Hence, it is of considerable practical interest to be able to alter the specificity of T cells, allowing them to recognize specific antigens on tumor cells, to make tumor-reactive T cells for current use. To achieve this, an approach has been developed by genetically engineering T cells to express chimeric receptors that will endow the T cells with a neo-specificity, variously referred to as "designer T cells", "universal receptor (UR) T cells", or "T-bodies". This is accomplished either by directly modifying the TCR or by linking ligand-binding domains derived from antibodies or other receptors with the signaling domains derived from the TCR-CD3 or other activating receptors. One of the currently favored immunoglobulin-T cell receptor (IgTCR) configurations is shown in Figure 3. However, many different designs have been examined, as shall be discussed. Designer T cells modified with these receptors recognize and kill tumor cells through the newly grafted chimeric receptors in a MHC-unrestricted fashion, which is of particular advantage when preparing reagents for wide clinical applicability.

For clinical application, patient T cells would be transduced with the chimeric genes, expanded ex vivo and then reinfused into the patients (Figure 4). In concept, cells would

traffick through the body with activation at the tumor site and induce tumor rejection, either by direct cytotoxicity and/or by causing a local inflammatory response [25]. This approach has the potential to treat a wide range of cancers by adoptive immunotherapy. In the past decade, T cells have been modified using this technology to create diverse kinds of chimeric receptors against a large number of different tumor models.

4.1. Structure of chimeric receptors

The chimeric receptor used to alter the specificity of T cells must possess two different functions: (1) to bind to a predetermined target antigen and (2) to activate the genetically modified T cells. Currently two types of chimeric receptors have been designed. One type is by grafting the TCR chains with a new antigen-binding domain. In this design, the activation signals are transmitted through the native TCR-CD3 complex. The other type is by directly linking the antigen-binding domain with a signaling chain that can deliver signal to the T cells necessary for T cell effector functions.

4.1.1. Antigen-binding domain

The antigen-binding domain can be either derived from antibody or TCR against the targeted antigen on the target cell surface, or derived from a ligand that can bind with a receptor on the target cell surface.

4.1.1.1. Derived from antibody

With current techniques, monoclonal antibody (mAb) against any kind of antigen can be readily derived, and such mAbs have been generated that are specific to different tumor antigens.

Kuwana et al was the first group to make immunoglobulin-T cell receptor (IgTCR) chimeric receptors. By using the variable regions from an anti-phosphorylcholine (PC) antibody and the constant regions from the TCR, they constructed two pairs of chimeric genes: V_L-C_β and V_H-C_α genes, and V_L-C_α and V_H-C_β genes [48], which functionally transmitted T cell signaling via the new PC specificity.

The specificity of antibodies for complex antigens (i.e., protein) typically relies on both the V_H and V_L regions, in which both the V_H and V_L are required to be incorporated into the chimeric receptors. Sometimes, however, the recognition specificity of an antibody may be contributed mainly by either the V_H or V_L region, typically with small molecules or haptens. In such a case, one single chain of V_H or V_L may be used for construction of the chimeric receptor. For example, the V_H regions from both the anti-trinitrophenyl (TNP) mAb [41, 49] and an anti-phosphorylcholine mAb [50] are such instances of single V molecules that have been used for construction of chimeric receptors.

The Fab fragment of antibody can also be used as antigen-binding domain for construction of chimeric receptors. Nolan et al [44] successfully expressed anti-CEA Fab-ε and Fab-ζ chimeric receptors on T cells that conferred CEA specificity whereas Yun et al [51] similarly modified T cells with a Fab for ganglioside GD3 specificity. In this, the

Fd chain (V_H-C_H1) was genetically joined directly to the ε or ζ chain, whereas the L

chain (V_L-V_H) was separately expressed in the T cells with successful assembly into

native Fab configuration. However, this strategy, like that of Kuwana et al, required the

expression of two separate constructs in the T cells, and is therefore technically more

cumbersome than the single chain constructs (below). Fab does have the advantage,

however, of preserving the native antibody affinity that may often be lost in single chain

Fv (sFv) constructs [52].

The design of a single-chain fragment of variable regions (sFv) [53, 54] in the

construction of the chimeric receptor overcomes the limitation of the two-constructs

strategy [55, 56]. The sFv consists of the V_H and V_L regions derived from an antibody

molecule that are joined via a flexible peptide linker. Ideally, the recombinant sFv retains

the antigen binding specificity and affinity of the parent antibody [57], but may also

suffer moderate [58] or major affinity loses [52]. Because the sFv are substantially more

convenient for T cell gene expression, however, they have come to be the predominant

constructs in the preparation of the chimeric receptors.

4.1.1.2. Derived from TCR

TCR recognize antigenic peptides presented in the context of major histocompatibility

complex (MHC) molecules. Similar to the use of the variable regions derived from

antibody to generate the chimeric receptor, the variable regions of the TCR may also be

used as recognition domains to generate chimeric receptors against TCR-specific peptide

antigens. These products are, however, subject to MHC restriction, i.e., applicable only

14

for individuals with a specific HLA type. By using the variable regions derived from the TCR from a melanoma-specific cytotoxic human T cell line, Willemsen et al generated a single-chain TCR recognition domain by linking the V_{α} and V_{β} with a linker as well as a two-chain TCR which expressed the V_{α} and V_{β} separately, finally linked to the CD3ζ signaling chain. These grafted chimeric receptors redirected T cells to recognize MAGE-A1 on HLA-A1$^+$ target cells [59]. HLA-A1 is represented in 40% of Caucasians, the primary disease population in melanoma, which means that even an MHC-dependent peptide can be widely targeted with a single, specific TCR construct for this group of subjects.

4.1.1.3. Derived from ligand

The binding domain derived from the ligand of a receptor may also be used to generate designer T cells that can specifically target receptor-expressing cells. Romeo et al fused CD4 with the TCR CD3 chains or with the Fc receptor γ chain. CD4 is the natural ligand for the gp120 HIV envelope protein. These receptor chimeras successfully redirected CD8$^+$ cytotoxic T lymphocytes (CTLs) to specifically recognize and lyse HIV-infected T cells [60]. Similarly, Altenschmidt et al used the binding domain from the human heregulin β1, which is the ligand to the erbB-3 and erbB-4 receptors, to generate a chimeric receptor that redirected T cells to recognize and kill erbB-3 and erbB-4 positive target cells [61]. Using the binding domain of IL-13, Kahlon and Jensen generated designer T cells that that recognize and kill IL-13R$^+$ malignant glioma and renal cell carcinoma [62].

4.1.2. Signaling domain

The antigen binding domain, however constructed, is ultimately linked to the cell signaling apparatus (1) by association with independent signaling molecules (e.g., modified TCR with the native CD3 complex) or (2) by direct genetic linkage to cellular signaling chains.

4.1.2.1. Association with signaling chain

T cells use the T cell receptor (TCR) for recognition of antigen on their target cells. The TCR associates non-convalently with signaling molecules of the CD3 complex (Figure 3). It is evident that specificity of T cells may be directed to new antigens by grafting the TCR α and β chains with different antigen specificities. In the very first IgTCR application, Kuwana et al successfully expressed two pairs of chimeric genes, V_L-C_β and V_H-C_α genes, and V_L-C_α and V_H-C_β genes in the murine EL4 T cell line. Increases in the concentration of cytoplasmic calcium ion were observed in the transduced EL4 cells when they contacted PC^+ bacteria [48].

Becker et al generated a hybrid IgTCR V_H-C_α gene using V_H from a digoxin-specific antibody. This hybrid V_H-C_α protein was expressed on a subset of T cells and assembled into a functional TCR complex. The modified T cells were activated with anti-idiotypic antibodies or digoxin [63]. Goverman et al demonstrated that a phosphorylcholine-specific V_H-C_α or V_H-C_β formed stable chimeric proteins in EL4 T cells. Both chimeric receptor chains associated with CD3 polypeptides in functional receptor complexes and responded to phosphorylcholine coupled to Sepharose beads. The V_H-C_α chimeric chain

associates with the EL4 β chain, while the V_H-C_β chimeric protein appears to form either a homodimer or a heterodimer with the native EL4 β chain [50]. Later, Brocker et al [64] generated a chimeric sFv-TCRβ and showed functional assembly with endogenous TCR/CD3 components. This allows the incorporation of new non-MHC-restricted ligand specificities into the intact TCR/CD3 complex that can exploit the full range of biological activities of the endogenous TCR signaling machinery.

4.1.2.2. Directly endowing signal domains with antigen-binding domain

The signaling domain delivers the signals for the activation of the designer T cells. Several signaling molecules that can deliver activation signals to T cells have been tested, among them FcεRI γ chain and CD3 ε, γ, δ, and ζ, as separate molecules and in complex. Of the CD3 chains, only ζ, the limiting chain in the complex, can be expressed alone on cell surfaces independent of other chains in the complex. All these molecules contain immune tyrosine activation motifs (ITAM) [65, 66], which upon phosphorylation serve as docking sites for other signal transducing molecules for T cell activation [67].

4.1.2.2.1. CD3 chains

After antigen recognition occurs by the αβ chains of the TCR, the signal is transmitted via the nonpolymorphic chains of CD3 to the intracellular signaling machinery [68].

CD3ε has a long history as the principal target for antibody-mediated T cell activation [69] and for redirected killing via bifunctional antibodies [70]. Nolan et al constructed anti-CEA sFv-ε and Fab-ε chimeric receptors. Such IgTCR-modified T cells showed

specific binding and activation, as demonstrated by IL-2 production on contact with immobilized CEA (Fab-ε, Fab-ζ) and on CEA⁺ tumor cells (Fab-ε) [44].

The CD3ζ chain is a nonglycosylated membrane protein that forms disulfide-linked homodimers as part of the CD3 complex. Each ζ chain contains 3 ITAMs and it plays a central role in the transduction of signals into the cells for the T cell activation. Several experiments showed that the intracellular portion of the CD3ζ chain is sufficient to induce activating signals after cross-linking of attached artificial extracellular domains [60, 71, 72]. Eshhar et al constructed chimeric genes by linking the sFv derived from an anti-TNP antibody with the CD3ζ chain. This chimeric gene was expressed as a functional surface receptor in a cytolytic T-cell hybridoma, and triggered IL-2 secretion upon encountering antigen and mediated non-MHC-restricted hapten-specific target cell lysis [55]. Brocker et al later confirmed this strategy in a separate experiment [56]. Nolan et al showed high surface expression of sFv-ζ that showed normal assembly with endogenous ζ into (sFv-ζ)(ζ) heterodimers as well as (sFv-ζ)₂ and ζ₂ homodimers [44].

4.1.2.2.2. FcεRI γ chain

The γ chain is the signal transducing subunit both of the high affinity IgE receptor (FcεRI) of mast cells and basophils, and of the low affinity receptor for IgG (FcεRIII), expressed primarily by macrophages and NK cells [73]. The γ chain is very similar in structure and function to the ζ chain in the CD3 complex and in fact can successfully replace the ζ chain in the CD3 complex in ζ-deficient T cells [74]. Chimeric receptors containing the signaling domain of the γ chain were initially designed for mediating

specific targeting of NK cells, but then were subsequently found to effectively transmit

stimulation signals in T cells as well [55, 75].

4.1.2.2.3. Other signaling molecules

Many tumor-bearing patients have been reported to have defective TCR-mediated

signaling events and therefore to exhibit impaired immune response [76]. To overcome

this limitation, Fitzer-Attas et al developed a new configuration of chimeric receptor in

which they used the cytosolic signaling domains of molecules that signal downstream of

TCR such as the protein tyrosine kinase (PTK). By connecting one of the PTK family

members, Syk, through a transmembrane stretch to a hinge spacer and the sFv domain,

they constructed a chimeric sFv-Syk receptor. A cytotoxic T cell hybridoma expressing

this chimeric receptor was efficiently stimulated to produce IL-2 upon interaction with

APC and specifically lysed appropriate target cells in a non-MHC-restricted manner [77,

78].

While different signaling molecules have been used for constructing the chimeric

receptors, among them the CD3 ζ chain has been the most potent signal transduction

molecule when expressed in isolation. It is reported that CD3 ζ chain was 300% more

potent than the CD3 ϵ and FcϵRI γ chain, both by IL-2 secretion and cytotoxicity [60, 79-

81]. On the other hand, the native ζ and ϵ chains, as well as their chimeric constructs,

function in T cells in the context of the full CD3 complex consisting of 10 ITAMs, in

which the signaling differences between the isolated CD3 ϵ and ζ chains disappeared

[44].

4.1.3. Spacer or hinge domain

An extracellular spacer or hinge region between the antigen binding domain and the transmembrane and signaling domains has been applied to extend the distance of the antigen binding domain from the cell membrane and to provide flexibility to the antigen binding domain in its interaction with cognate antigen. A hinge was first shown to be important with CD3ζ chain, which has a minimal extracellular domain. The presence of hinge markedly improved expression, ligand binding and signaling activity of the chimeric receptors [82, 83]. The CH2/3 constant domain of human IgG [84], the C_H1 constant domain of Fab [44] and the extracellular domains of CD8 and CD7 have been used for this purpose [85]. Ren-Heidenreich et al found that T cells transduced with the chimeric receptor with a CD8α hinge displayed specific lytic activity that was twofold greater than that of chimeric receptor without the hinge and also produced significantly more cytokines [86]. On the other hand, the MOv18 sFv-γ chimera functions and expresses well without an intervening hinge spacer (Huw, personal communication).

The critical nature of this distance effect was further suggested in studies by Nolan et al [44]. The CD3ε chain was coupled with an sFv and a Fab, whereas CD3ζ was coupled with Fab or with sFv with an intervening CD8α hinge (Figure 5). It was thought that the C_H1-C_L domain of the Fab would provide the needing spacing of the Ag-binding domain (Fv) above the membrane with the CD3ζ signaling chain, but that the Ig-like extracellular domain of CD3ε would provide this spacer function to allow efficient targeting with sFv when directly conjugated. While all four constructs activated equally well with

20

immobilized CEA antigen, the sFv-ε alone failed to bind CEA+ tumor cells and activate. Among the constructs, the sFv-ε was estimated from molecular models to have the shortest projection from the membrane (47Å versus 65Å for Fab-ζ, the second shortest) (K. Johnson and R. P. Junghans, unpublished analyses). The antigen epitope on CEA is in the second domain of seven from the membrane. The importance of adequate intracellular distance due to electrorepulsion between negatively charged cellular membranes has been stated [87] and achieving this distance with or without spacers may be different with different antigen-chimeric receptor pairs.

4.2. Effector cell types

The basic principle of the chimeric receptor strategy for adoptive immunotherapy is to graft effector cells with neo-antigen specificity and then to infuse these back into patients to eradicate the antigen-expressing target cells. The most potent candidate effector cells are autologous peripheral blood T lymphocytes stimulated by anti-CD3 antibody alone or in combination with anti-CD28. Hwu et al developed another system by using TILs, which are tumor-reactive T cells isolated from tumor sites [41, 88]. Cytotoxic T cell lines such as the human TALL-104 T cell line are a further example, which has been approved for clinical trial [89] and may also be a good candidate to be modified with the chimeric receptor and used in an analogous manner.

Apart from T cells, chimeric receptors may also be used to redirect other cell types. NK cells are known to have an inherent capacity to target many types of tumor cells, but lack adequate specificity for successful in vivo therapy. Bach et al reported that NK cells

21

transfected with chimeric receptor genes can specifically lyse tumor cells [90]. Wang et al reported that recipients of bone marrow cells transduced with chimeric receptor genes exhibited significant antitumor activity in vivo. In vivo depletion of T cells in the reconstituted mice did not affect antitumor activity, suggesting that other non-T immune cells expressing the chimeric receptor gene may play an important role in tumor rejection [91]. Roberts et al reported specific lysis by neutrophils expressing chimeric receptors [80]. It is anticipated that chimeric receptors would also function in activating monocytes, macrophage, and other cell types [92].

For some applications, chimeric receptor modification of hematopoietic stem cells (HSC) may be preferable to modification of terminally differentiated T cells. The self-renewing nature and transplantability of hematopoiesis would allow the permanent generation of a stable population of tumor-specific effector cells. Hege et al modified murine HSC with a human immunodeficiency virus (HIV) envelope-specific chimeric receptor consisting of the extracellular domain of human CD4 linked to the ζ chain of the T cell receptor (CD4-ζ). After transplantation into immunodeficient SCID mice, sustained high level expression of CD4-ζ was observed in circulating myeloid and natural killer cells. CD4-ζ-transplanted mice were protected from challenge with a lethal dose of a disseminated human leukemia cell line expressing HIV envelope [93].

4.3. Monitoring chimeric receptor expression and enriching modified cells

Antibodies that can recognize the chimeric receptors expressed on the modified cells are very useful tools for monitoring the chimeric receptor expression, for enriching the

receptor expressing cells by panning or cell sorting, and for testing the function of the chimeric receptor. The chimeric receptor's expression can be detected by anti-idiotypic antibodies for the sFv (Figure 6) [24], antibodies recognizing the spacer region such as the CH2/CH3 region of human IgG, or antibodies against a tag peptide inserted into the chimeric receptors. Soluble ligand/antigen may be similarly applied for panning or, when appropriable conjugated, for detection of expression

4.4. Function of designer T cells

4.4.1. Expression

For functional expression on the T cell surface, the chimeric molecules when appropriately expressed will assemble as part of a functional receptor complex, as supported by several lines of evidence [50, 94]. In this manner, the chimeric receptors that have successfully assembled will transduce signals to the modified T cells in the manner of the parent receptor.

4.4.2. Binding

The formation of conjugates between the effector T cells and their target cells is a prerequisite for T-cell dependent activation and cytolysis. Upon contact with the target cells, the chimeric receptor-modified T cells can bind tightly with the target cells as shown in Figure 7 by Yun et al [51].

4.4.3. Activation

Once T cells bind specifically with the target cells by TCR, the T cells will be activated to secrete cytokines to regulate the immune reaction. With co-culturing of chimeric receptor modified T cells with their target cells, increased production of several cytokines have been recorded such as IL-2 (Figure 8) [44, 56], IL-3 [56], IFN-γ [95, 96] and GM-CSF [75]. It is noted, however, that the IL-2 assays are typically in the presence of phorbol ester (PMA) that bypasses the need for co-stimulatory signals of normal IL-2 secretion [97, 98]. As such, this assay is a sensitive measure of TCR activation but not of authentic IL-2 secretion.

4.4.4. Cytotoxicity

The cytolytic function of chimeric receptor-modified T cells is the most important property for the deployment of T cells in adoptive immunotherapy. The engineered T cells lyse their target cells in chimeric receptor-specificity dependent and MHC non-dependent fashion [55]. In addition, Weijtens et al [99], Nolan et al [44] and Yun et al [51] found that the engineered T cells could recycle their lytic activity by cycling between target cells, with multiple killing events per T cell (Figure 9).

4.4.5. In vivo tumor suppression

Using in vivo model systems, Moritz et al demonstrated that the growth of ErbB2-transformed cells in athymic nude mice was retarded by adoptively transferred cytotoxic T lymphocytes (CTLs) modified with a chimeric receptor specific for ErbB2 receptor-expressing tumor cells (Figure 10). They further demonstrated that only T cells expressing the chimeric receptor gene homed to the ErbB2-expressing tumors; nontransduced parental T cells did not [100]. Hwu et al reported that lymphocytes

transfected with a chimeric receptor of anti-folate binding protein specificity, were shown to eliminate experimental tumors in mice (Figure 11) [101]. Altenschmidt et al treated tumor bearing mice with transduced T cells and found that the adoptively transferred sFv(FRP5)-ζ-expressing T cells caused total regression of ErbB-2-expressing tumors [102]. In a recent study, Darcy et al reported that in vivo specific tumor cell cytotoxicity was mediated by gene-modified $CD8^+$ T cells. Furthermore, using gene-targeted and chimeric receptor-transduced donor CTL, perforin and IFN-γ were demonstrated to be critical for the eradication of colon carcinoma in mice [96].

4.5. Tumor antigens as designer T cell targets

It has been controversial for decades whether tumor "specific" antigens exist. Several tumor antigens that can induce T-cell or humoral immunity have been cloned from different tumor models [103]. Those antigens are either mutated proteins or they may be normal self-proteins that are over-expressed in tumor. How to manipulate effective T cell immunity against these tumor antigens has been a major objective of recent tumor immunology strategies. Retargeting T cells against tumor antigens by genetically engineered T cells with chimeric receptors is one such approach. However, the T cells may also be redirected at proteins that are expressed in normal tissues when treating metastatic tumors (e.g., prostate membrane specific antigen on prostate).

In this chimeric receptor strategy, it is evident that the targeted antigen must be membrane-bound, expressed on the surface of tumor cells. Using this technology,

25

redirected T cells have been developed against a large number of predefined tumor

targets as listed in Table 1.

4.6. Clinical trials

To date, patient experience with designer T cells has been limited, and most studies are

still unpublished. Table 2 lists trials that are current or soon to be initiated. A CD4-ζ

chimera [127] was applied in HIV with doses to 3×10^{10} cells in 24 patients, 11 with IL-2

infusion × 1wk, with good tolerance and decreased viral load in the mucosal

compartment [128]. Studies of ovarian carcinoma with an anti-MOv18 chimera [101]

applied 18 doses to a maximum of 5×10^{10} cells + IL-2 in eight patients without toxicity

or clinical response (P. Hwu, personal communication). An anti-TAG72 IgTCR was

applied in colorectal cancer, with dose to 10^{10} cells in ten patients, was well tolerated

without clinical responses [129] (K. Hege, personal communication). The initial report

indicated responses after treatment by decline of TAG72 in several patients. However,

this was lately shown to be due to an anti-idiotype antibody response in patient sera that

interfered with an ELISA assay to give a false impression of decreased TAG72 (K. Hege,

personal communication). Our own on-going Phase I tests applied 24 doses of anti-CEA

IgTCR up to 10^{11} cells in seven patients, with adequate tolerance and minor responses in

two patients [130]. In general, treatment with IgTCR-modified T cells has been well

tolerated with suggestions of efficacy, but until now without durable, meaningful

responses. The outcome of these several on-going and pending studies will be awaited

with interest, and will undoubtedly yield crucial information to guide future

modifications and improvements in the best traditions of translational research. Some of

these needed improvements are already becoming evident from the presently available phase I data, as described in the following section.

5. Conclusions and Future Directions

The challenge for using designer T cells for adoptive immunotherapy is how to enhance and sustain the survival and expansion of the modified T cells in the tumor sites. Currently, the chimeric receptor modified T cells are ex vivo activated T cells. One of the limitations for this kind of adoptive immunotherapy has been that TCR ligation by the activated T cells usually leads to activation-induced cell death (AICD) [106, 131]. Although the modified T cells execute their effector functions to kill the target cells, they will ultimately undergo AICD after contacting the target cells. How to prevent the modified T cells from AICD when they conjugate with their targets is a key to the next generation of designer T cells to make this treatment more successful. The knowledge obtained from current research developments in the processes of T cell activation and apoptosis will play a crucial role in directing the development of future modifications of this new type of therapy.

According to the generally accepted two-signal requirement model for T-cell activation, the functional outcome of TCR ligation varies, depending on the presence or absence of additional costimulatory signals delivered to the T cell via APC [97, 98]. Antigenic stimulation can lead either to a productive immune response characterized by proliferation, differentiation, clonal expansion and effector function or, in the absence of appropriate costimulation, to a state of long-lasting antigen-specific unresponsiveness

termed anergy, or to cell deletion [132]. CD28 is one of the crucial receptors on the T cell surface to receive costimulatory signals delivered by B7-1 and B7-2 from the APC, with impact on multiple aspects of T-cell physiology. CD28 ligation enhances the magnitude and duration of T-cell responses; induces the anti-apoptotic gene Bcl-X_L; increases cytokine secretion, particularly IL-2; enhances cell adhesion; facilitates reorganization of the T-cell plasma membrane upon binding to an APC; prevents energy induction; and supports germinal center formations [133].

Several groups have explored the possibility to incorporate a costimulation signal into the IgTCR modified T cells [25, 106, 112, 134, 135]. Alvarez-Vallina et al generated T cells that express both sFv-CD3ζ and sFv-CD28 and they demonstrated that antigen-specific co-stimulatory signals could synergize with signals mediated through chimeric CD3ζ chains to secrete maximal levels of IL-2 [134]. Beecham et al showed that in addition to IgTCR signals, CD28 costimulation is required to induce expansions of previously activated peripheral blood mononuclear cell-derived T cells. Signals from IgTCR alone could induce transient cell division, but they could not induce the prolonged polyclonal expansions that are characteristic of native immune responses. CD28 costimulation also suppressed the deletion of tumor-reactive subclones by AICD [106]. Eshhar et al modified human PBL with a tripartite construct consisting of a sFv for TNP linked to part of the extracellular domain and the membrane spanning regions of the CD28 and joined at its cytoplasmic terminus to the γ signaling chain. Upon stimulation with immobilized TNP, the chimeric receptor-expressing T cells produce IL-2, underwent specific proliferation in the absence of exogenous IL-2, and effectively lysed target TNP+ cells.

However, in the presence of antigen excess, AICD of the modified T cells was not prevented by the presence of CD28 in the signal receptor [25]. Whereas other signals clearly will be important in their sustained growth, the ultimate configuration(s) of designer T cells for therapeutic success is still to be determined.

Genetic engineering of T cells with chimeric receptors attempts to combine the specificity of antibodies with the efficient cytotoxic effector properties of T cells. This and other areas of research will shape future generations of designer T cells in the evolving application of these new technologies. There is reason for optimism that this path will lead to important therapeutic impact in many cancers for which options are presently woefully lacking. The next 5-10 years are likely to produce dramatic gains in immunotherapeutic modalities generally, and in targeted cellular strategies specifically.

Figure Legends

Figure 1. Retroviral vector action. A packaging cell line (or helper cell line) contains separately expressed viral genes for enzyme and structural proteins that produce empty virions. The gene of interest ("transgene") is cloned into a retroviral vector DNA which is transferred into the packaging cell line, creating a vector producer cell (VPC). The RNA transcripts from the vector DNA possess a packaging signal (ψ+) that is recognized by the capsid proteins and causes RNA to be packaged thus creating complete viral particles. These viral supernatants are used to infect the T cell, where the viral RNA is copied to DNA by the virally encoded Reverse Transcriptase. The viral DNA is then integrated into the T cell genome for stable expression of the transgene. (Modified from reference [18], with permission).

Figure 2. Retroviral vectors used to transduce TILs with genes for neomycin phosphotransferase (Neo^R) and TNF. Arrow indicates gene transcription from LTR promoter. SV, SV40 virus promoter; TM, transmembrane. (From reference [29], with permission).

Figure 3. Structure of an IgTCR. The T cell receptor/CD3 complex (TCR) is comprised of six proteins: α, β, γ, δ, ϵ and ζ. The structure of the $\alpha\beta$ receptor chains is analogous to the Fab portion of an antibody (Ab) with variable (V) and constant (C) domains. γ and ϵ also have Ig-like extracellular domains. Boxes in the cytoplasmic domains represent signaling motifs in all chains except α and β. Antibody V regions are engineered to

30

create an sFv and then linked to the signaling portion of ζ, in this IgTCR representation, with an intervening spacer derived from the hinge portion of CD8α. (From reference [44] with permission).

Figure 4. Scheme for preparation of designer T cells for patient treatment. Peripheral blood mononuclear cells are harvested from the patient, and T cells are activated by anti-CD3 antibody treatment for 2-3 days. Subsequently, T cells are infected with IgTCR retroviral supernatant to express the IgTCR transgene. Autologous chimeric T cells are expanded ex vivo and infused back into the patient, who is then observed for anti-tumor effect.

Figure 5. Representation of the IgTCR molecules. sFv-CD8α hinge-ζ, Fab-ζ, sFv-ϵ and Fab-ϵ are shown in the context of complete TCR complexes. The distance of the antigen-binding domain from the cell membrane is different for each four constructs. (From reference [44], with permission).

Figure 6. Detecting IgTCR expression. Activated human T cells were infected with anti-CEA IgTCR vector, stained with either PE-labeled anti-idiotype antibody (solid lines) or with negative control antibody (dotted lines). (From reference [24], with permission).

Figure 7. Cell conjugation of IgTCR+ T cells with GD3+ melanoma cells. After co-incubation of nonadherent T cell effectors (round shaped cells) and adherent tumor cell targets (spindle shape) on plates, unbound T cells were removed by washing. (A)

Unmodified T cells were nearly completely washed away, showing only the tumor cells left in plate. (B) IgTCR+ modified T cells bound tightly to GD3+ melanoma cells (From reference [51], with permission).

Figure 8. Anti-CEA designer T cells secrete IL-2 on binding CEA+ tumor cells. Jurkat IgTCR+ transduced cell line was stimulated with various stimuli and the supernatants were harvested and tested for IL-2 concentrations. Stimulators were: BSA; CEA; CEA-negative colon cancer cell line (MIP-101); CEA-positive colon cancer cell line (MIP-CEA). IL-2 levels were standardized to OKT3-stimulated secretion. (From reference [44], with permission).

Figure 9. Cytotoxicity of IgTCR-modified normal human T cells against antigen-expressing targets. MIP-101 (CEA-) and MIP-CEA (CEA+) tumor cells were seeded at 10^5 cells per well, to which IgTCR-modified CD8+ CTLs were added at an E:T ratio of 0.15:1, and counted daily for viable tumor cells. (From reference [44], with permission).

Figure 10. Cytotoxicity and antitumor activity of sFv/ζ-transduced CTLs against human ERBB2-expressing target cells. (A) In vitro 8 hr-cytotoxicity assay (LDH release). sFv/ζ-transduced CTL lysed ErbB2-transfected NIH 3T3 fibroblasts (■) or ErbB2-transfected HC11 breast epithelial cells (●) at varying effector:target (E:T) ratios. Control, unmodified CTLs were unable to lyse either cell line (○, □). (B) In vivo co-administration of transduced CTLs and tumor cells suppressed tumor formation for up to 8 days. ErbB2+ tumor cells were mixed with sFv/ζ-transduced CTLs (■) or nontransduced CTLs

(□) at a ratio of 10:1 and injected subcutaneously. Tumor cells without CTLs (▲) are shown as a control. (C) In vivo antitumor activity with delayed injection of sFv/ζ-transduced CTLs. Balb/c mice were inoculated subcutaneously with ErbB2+ tumor cells. On days 4 and 5, when tumors were palpable, nontransduced CTLs (□) and transduced CTLs (■) were injected intravenously through the tail vein. IL-2 was administered intraperitoneally on days 4, 5 and 6. Tumor cells without (▲) and with (△) IL-2 are shown as controls. The transduced CTLs delayed growth of tumor cells, but without curing the mice. (From reference [100], with permission).

Figure 11. In vivo activity of MOv-γ-transduced TIL against human ovarian carcinoma (IGROV) cells over-expressing folate binding protein (FBP). Three days after i.p. injection of nude mice with IGROV cells, mice were treated with saline solution (HBSS), TILs that were nontransduced (NV-TIL), transduced with the anti-FBP chimeric MOv-γ receptor (MOv-TIL) or transduced with control irrelevant chimeric receptor (TNP-TIL). Mice treated with MOv-TIL had significantly increased survival (P<0.002) compared to other groups. (From reference [101], with permission).

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Table 1. Tumor antigens as targets for designer T cells

Antigen	Tumor	Reference
Angiogenic endothelial cell receptor: Kinase insert domain-containing receptor (KDR)	Tumor neovasculature	[104]
Carcinoembryonic antigen (CEA)	Colorectal, breast, lung and other cancers	[24, 44, 81, 95, 96, 105-108]
CD20	B-cell lymphoma	[109]
CD30	Hodgkin's lymphoma	[110, 111]
CD33	Leukemia	[112]
CD44	Pancreatic carcinoma	[113, 114]
EGP40	Colorectal cancer	[115]
Epithelial glycoprotein 2 (EGP-2)	Many human carcinomas (breast, colon, lung, renal, pancreatic)	[86]
ErbB-3 and ErbB-4		[61]
Folate binding protein (FBP)	Ovarian carcinoma	[75, 91, 101, 101, 116]
G250	Renal cell carcinoma	[99, 117, 118]
GD3	Melanoma	[51]
High molecular weight-melanoma associated antigen (HMW-MAA)	Melanoma	[119, 120]
Lewis[y]	Several human carcinomas	[121]
MAGE-A1/HLA-A1	Melanoma	[59]
Neu/HER-2/ErbB-2	Adenocarcinomas	[61, 100, 102, 122]
Prostate specific membrane antigen (PSMA)	Prostate cancer	[123, 124]
TAG72	Gastrointestinal adenocarcinoma and pan-adenocarcinoma	[84, 125, 126]

Table 2. Clinical trials with chimeric receptors redirected T cells.

Date initiated	Phase	Disease	Antigen	Structure	Location	Investigator
1996	I	Ovarian cancer	FBP	sFv-γ	NCI	Hwu
1997	I	HIV	gp120	CD4-ζ	Multi	Hege
1997	I	Adenocarcinoma	TAG72	sFv-ζ	Stanford	Hege
1998	I	Adenocarcinoma	CEA	sFv-ζ	Harvard	Junghans
2000	I	Lymphoma	CD19	sFv-ζ	City of Hope	Jensen
2002 (pending)	I	Renal cell carcinoma	G250	sFv-ζ	den hoed CC	Bolhuis
2002 (pending)	I	Melanoma	GD3	sFv-ζ	Harvard	Junghans

Ψ+ TRANSCRIPTS
PACKAGED

RNA TRANSCRIPT

VECTOR

LTR Ψ+ cDNA LTR

gag pol env

PACKAGING CELL

Ψ− TRANSCRIPTS
NOT PACKAGED
(EMPTY VIRIONS)

cDNA

Reverse
transcription

TARGET CELL

cDNA

Integrated provirus

A

LTR   Neo-R   LTR

B

LTR   TM   TNF   SV   Neo-R   LTR

Antibody
(immunoglobulin)

T cell receptor (TCR)

IgTCR

gene
therapy

TCR

CD3

αβ

γε δε

ζζ

Gene-modified IgTCR

CD3

αβ

γε δε

ζζ

sFv-ζ

VL VH   VH VL   αβ

γε   δε

ζ   ζ

Fab-ζ

VH   VH   αβ
VL   CH1 CH1   VL
Cκ   Cκ

γε   δε

ζ   ζ

sFv-ε

αβ
VL VH   VL VH

ζ   ζ   γ   ε   δ   ε

Fab-ε

αβ   VL VH   VL VH
     Cκ CH1   Cκ CH1

ζ   ζ   γ   ε   δ   ε

Figure: Flow cytometry histogram. Y-axis "Cell Number" with values 0, 125, 250, 375, 500. X-axis "IgTCR+" with log scale values .1, 1, 10, 100, 1000. Legend: Negative Control (UPC) shown as dotted line, Anti-Idiotype (WI2) shown as solid line.

% Surviving (y-axis)

100%
80%
60%
40%
20%

MOv-TIL

TNP-TIL

HBSS

NV TIL

0 10 20 30 40 50 60 70 80 90 100 110 120

TIL →

Days after I.P. IGROV

Coupling CD28 Co-Stimulation to Immunoglobulin T-Cell Receptor Molecules: The Dynamics of T-Cell Proliferation and Death

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Summary: Immunoglobulin T-cell receptor (IgTCR) molecules are potentially potent immune response modifiers because they allow T cells to bypass tolerance. Tolerance to self antigens has been one of the major barriers to the development of effective adoptive immunotherapies for treating cancer. In vitro studies in several laboratories have shown that cross-linking IgTCR molecules with the target antigen leads to cytolytic activity, cytokine release, and T-cell proliferation in model systems. However, many of these studies have used established T-cell lines rather than normal T cells or indirect assays of cytotoxicity, proliferation, and cytokine release. We have sought to establish the validity of these model systems while developing more effective adoptive immunotherapies using normal human T cells. In the present study the activation of T-cell proliferation after IgTCR cross-linking was evaluated. The results show that, in addition to IgTCR signals, CD28 costimulation is required to induce expansions of normal peripheral blood mononuclear cell–derived T cells. Signals from IgTCR alone can induce transient cell division, but they do not induce the prolonged polyclonal expansions that are characteristic of native immune responses. Very strong IgTCR signals could circumvent the CD28 requirement, but only at levels that are unlikely to be physiologically relevant. CD28 costimulation also suppressed the deletion of tumor-reactive subclones by activation-induced cell death. These studies confirm the importance of CD28 costimulation to the proliferation of IgTCR-modified human T cells, a key feature of an effective, reconstructed antitumor response. Key Words: Immunoglobulin T-cell receptor—CD28—Activation—Proliferation—Clonal selection.

The hallmarks of an effective cellular immune response are 1) cytolytic activity (cytotoxic T lymphocytes), 2) T-cell proliferation, and 3) cytokine release. Each of these T-cell functions plays an important role in determining the overall success of the response. The functions of cytotoxic T lymphocytes are responsible for physically eliminating pathogens or infected cells. Cytokines have a wide range of functions, but they generally promote the growth or differentiation of different immune cell subsets. Proliferation is crucial to the expansion of responsive T-cell clones in numbers adequate to eliminate an infectious agent.

Immunoglobulin T-cell receptors (IgTCR) are chimeric molecules that consist of the antigen-binding portion of an antibody fused to one of the chains of the TCR. The binding properties of IgTCR molecules provide several advantages for adoptive immunotherapy to treat cancer. First, the large number of possible antibody combinations allows T-cell specificity to be assigned to virtually any antigen. Most tumor antigens are self proteins, and T cells that can recognize these self antigens have either been deleted or have become tolerant. Thus, the selection of the appropriate antibody specificity allows IgTCR molecules to bypass tolerance. A second important advantage is that IgTCR-modified T cells have

greater autonomy than normal T cells, because IgTCR-modified cells are activated directly by the immunologic target rather than through a complex process of antigen presentation by accessory cells. Finally, antigen binding is much stronger for IgTCR molecules than for the normal TCR, because antibody affinities are several orders of magnitude greater than that of a typical TCR molecule. Higher binding affinity may allow IgTCR molecules to function independently of the adhesion–costimulatory molecules usually required to facilitate normal TCR binding.

Many studies have examined the ability of IgTCR molecules to activate T cells (1–16). Direct cross-linking of the chimeric receptor by the target antigen leads to potent antigen-specific cytolytic activity (3–13,15), and the release of cytokines such as interleukin-2 (IL-2) (2, 3,5,7,13,16), interferon-γ (7), granulocyte-macrophage–colony-stimulating factor GM-CSF (6,10), and tumor necrosis factor-α (1,9). In addition, two studies (7,8) have reported that IgTCR cross-linking induces T-cell proliferation. The ability of these molecules to single-handedly induce all major T-cell functions suggests that IgTCR activation may depend less on the participation of costimulatory or adhesion molecules such as CD28, LFA-1, and CD2.

When normal T cells encounter antigen, they respond in one of several ways. These reactions can include complete activation, partial activation, anergy, or the induction of apoptosis through a process called activation-induced cell death (AICD). Which reaction occurs is determined by the types of signals the cell receives. In addition to TCR ligation (signal 1), T cells usually require one or more costimulatory signals to become fully activated. The most well defined of these is delivered through the CD28 receptor (signal 2) [reviewed in Sperling and Bluestone (17) and Boussiotis et al. (18)]. When T cells do not receive CD28 costimulation, a state of unresponsiveness or anergy is usually induced (17,18). However, increasing the strength of signal 1 can lead to a reduced dependence on CD28 costimulation, with enhanced proliferation and IL-2 production in the absence of CD28 costimulation (19–23).

One implication of these studies is that the greater binding affinity of IgTCR molecules may allow them to bind tightly to antigen and transmit very strong signal 1. We examined the hypothesis that such strong IgTCR signals might reduce the dependence on CD28 and be sufficient to independently induce proliferation in normal T cells. In the current study, proliferation was examined after stimulation with different combinations of IgTCR and CD28 activation signals. The results confirm

the central role of CD28 (signal 2) for sustained ex vivo proliferation that can be superceded only in part by strong signal 1. These studies increase our understanding of the key immune elements necessary for designing effective antitumor T-cell gene therapies in humans.

MATERIALS AND METHODS

Retroviral Vector and Vector Producer Cells

The construction of anti-carcinoembryonic antigen (CEA) IgTCR genes has been previously described (15,16). The current studies were conducted with an IgTCR gene containing sequences encoding the heavy and light chain variable regions (joined by a flexible linker) from the humanized MN14 antibody fused to sequences encoding the ζ-chain of the human TCR (16). The antibody and ζ-chain sequences are separated by the hinge region of CD8α. The anti-CEA sFvζ construct was inserted into the MFG retroviral vector backbone, as previously described (15). Retroviral vector supernatant was produced using the PG13 producer cell line (15).

The pLNSX-hB7.1 vector (provided by Dr. Lieping Chen, Mayo Clinic, Rochester, MN, U.S.A.) contains a cDNA copy of the human B7-1 gene inserted into the LNSX vector backbone. LNSX-hB7.1 vector producer cells were constructed by transfecting the vector into GPE86 ecotropic helper cells and using the transient viral supernatant to infect the PA317 packaging cell line. PA317 cells were then selected with G418 for 10 days, after which viral supernatant was harvested and used to transduce the MIP-CEA and MIP-101 tumor cell lines.

Lymphocyte Transduction and Culture

Peripheral blood mononuclear cells from normal blood were cultured and transduced, as previously described (15). after transduction, T cells were expanded in growth media (AIMV supplemented with 10% fetal calf serum, and 300 U/mL recombinant IL-2) until the cells reached plateau phase and the rapid cell proliferation caused by the initial OKT3-induced activation had ceased. T cells were then used in proliferation assays. Individual preparations of T-cell cultures varied slightly but generally consisted of approximately 40% to 50% IgTCR-positive cells, of which approximately 60% were CD4+ and 40% CD8+ (data not shown).

Tumor Cell Lines and Transductions

MIP-101 is a poorly differentiated human colorectal cancer cell line that does not express CEA (24). The

MIP-CEA cell line was derived by transfecting MIP-101 with a full length cDNA encoding the human CEA gene (25). Tumor cells were cultured as previously described (15). Tumor cell lines expressing the human B7.1 gene (MIP-101.B7 and MIP-CEA.B7) were constructed by infecting parental MIP-101 and MIP-CEA cells, respectively, with a B7.1-containing retroviral vector (LNSX-hB7.1). Expression of the CEA and B7.1 proteins in tumor cells was confirmed by staining the cells with P-phycoerythrin (PE)–conjugated hMN14 and fluorescein isothiocyanate–conjugated anti-human B7.1 antibody, and then analyzing the cells by fluorescence-activated cell sorting.

Antibodies and Flow Cytometric Analyses

Humanized MN14 (hMN14) antibody and its anti-idiotype antibody, WI2, were obtained from Immunomedics (Morris Plains, NJ, U.S.A.). WI2 is designated as anti-IgTCR in the text. DAB9.3 is a mouse antibody against the human CD28 receptor (provided by Dr. Carl June, University of Pennsylvania, Philadelphia, PA, U.S.A.). DAB9.3 was used as a binding analog of the human B7 antigens in experiments using plate-bound antigens. OKT3 (Ortho Biotech, Raritan, NJ, U.S.A.) is a mouse antibody directed against the normal human TCR and was used as a binding analog for antigen-MHC complexes in experiments using plate-bound antigens. UPC-10 (Sigma Chemical, St. Louis, MO, U.S.A.) is a mouse antibody with binding specificity for β-2–6 linked fructosan and was used as a negative control staining antibody in experiments using mouse antibodies. HAT (Hoffmann-La Roche, Nutley, NJ, U.S.A.) is a humanized anti-Tac antibody and was used as a negative control staining antibody for experiments using hMN14. Anti-human B7.1 antibody was obtained from Ancell Corporation (Bayport, MN, U.S.A.). Fluorescein isothiocyanate (FITC) and PE–labeled antibodies against different human T-cell antigens (CD4, CD8) and against mouse and human Fc were obtained from Caltag Laboratories (Burlingame, CA, U.S.A.). All antibody staining reactions were performed using standard methods (26). Fluorescence intensity was measured using a Coulter (Miami, FL, U.S.A.) EPICS Profile II flow cytometer.

T-Cell Proliferation Assays

Proliferation Assays Using Plate-Bound Antigens

Ninety-six–well tissue culture plates were coated with antibodies by incubation overnight in 0.1 mol/L sodium bicarbonate buffer (pH 7.0), followed by washing with fresh media. Cells were added to replicate wells so that an undisturbed well could be harvested on each day for which cell counts were to be performed. At specified times, T cells were harvested from wells by vigorous pipetting to remove bound cells from the plate surface and to create a single cell suspension. Viable cells were quantified by trypan blue exclusion. When possible, a minimum of 100 cells were counted for each test sample. Individual wells were counted from two to nine times, depending on the cell density. For wells that contained a high density of cells, two duplicate counts were performed. In cases in which extensive cell death or a lack of proliferation made it difficult to obtain more than 100 cells for counting, a minimum of nine counts were done. These cell counts were averaged to obtain the total number of viable cells for each time point and treatment group in a given experiment. Cell counts from repeated independent experiments were averaged for each time point and treatment group, and the number of viable cells plotted as a function of time.

Proliferation Assays Using Tumor-Bound Antigen

Tumor cell lines were seeded into Falcon 1013 150-mm tissue culture plates and incubated overnight at 37°C to allow time for the cells to adhere and begin proliferating. Twenty-four hours later, IgTCR-modified T cells were added to the culture plates, and the plates were returned to incubation at 37°C. During tumor cell/T-cell coincubation periods, T cell growth media was used to maintain the activational state of the T cells. Both MIP-101 and MIP-CEA proliferate normally in this media. T cells and tumor cells were counted every few days by trypsinizing the plates to create single cell suspensions, mixing an aliquot of the suspension with trypan blue, and counting the cells as described above. Both MIP-101 and MIP-CEA cells are two to three times larger than human T cells and can be distinguished readily from T cells using light microscopy.

RESULTS

The central objective of these studies was to determine which activation signals are required to induce proliferation in normal human T cells. The premise that IgTCR signals alone are sufficient for proliferation is supported by the many in vitro studies showing that IgTCR cross-linking activates all major T-cell responses (1–16). However, these previous reports of IgTCR-induced proliferation are based on increased ^{3}H-thymidine incorporation (7,8) in IgTCR-stimulated cells. ^{3}H-incorporation assays measure DNA replication transiently, and even high lev-

els of ^{3}H-incorporation may not accurately reflect the logarithmic expansion of T cells that occurs during a native immune response. Furthermore, when measured early in the response, even anergizing signals that lead to abortive proliferative responses can induce levels of ^{3}H-incorporation that are similar to those obtained with nonanergizing signals (27). In the current studies, we used direct cell counting methods rather than ^{3}H-incorporation to measure proliferation. Our overall goal in these studies was to evaluate proliferation from an immunologically relevant perspective. This requires the ability to distinguish between true logarithmic expansions of cells and abortive proliferative responses that may involve DNA replication but which ultimately end in AICD. Direct cell counting methods are more effective than ^{3}H-incorporation assays for accomplishing this.

As a model, we have used an IgTCR molecule directed against CEA (15,16). To distinguish between transient replication and true logarithmic expansions, we used direct cell counting methods over long periods of time. To analyze proliferation under physiologically relevant conditions, we used peripheral blood mononuclear cell–derived T cells rather than transformed T-cell lines, and antigenic stimuli were displayed on the surface of tumor cells. T cells used in the current studies were cultured for approximately 20 to 30 days before the start of any experiments so that the cultures would reach plateau phase and cease the rapid proliferation caused by the initial anti-CD3 activation. At this stage, cultures of peripheral blood mononuclear cell–derived T cells are generally in decline and consist of two populations of cells: 1) dead and dying cells and 2) viable, resting cells. T cells that remain viable in these plateau cultures have generally returned to a resting state (in a single cell suspension of small rounded cells), but still can become reactivated when stimulated with antigen [see Beecham et al. (15)].

Immunoglobulin T-Cell Receptor Expression in Normal Peripheral Blood T Lymphocytes

IgTCR expression in cultures of primary human T lymphocytes was measured by flow cytometric analysis. After two rounds of infection, approximately 40% to 50% of the T cells expressed the anti-CEA IgTCR gene (Fig. 1). The ratio of CD4+ and CD8+ cells in different preparations of T cells was approximately 60% CD4 and 40% CD8 (data not shown). Expression of the anti-CEA IgTCR gene was stable in long-term cultures of primary human T cells, remaining virtually unchanged for more than 2 months (data not shown). All T-cell experiments described in this report were performed using unselected populations of T cells with proportions of IgTCR-negative and IgTCR-positive cells similar to the population shown in Figure 1.

Immunoglobulin T-Cell Receptor–Mediated Stimulation of T Cells Results in Cell Death Rather Than Proliferation

Initial experiments focused on determining whether stimulating T cells with only IgTCR signals would in-
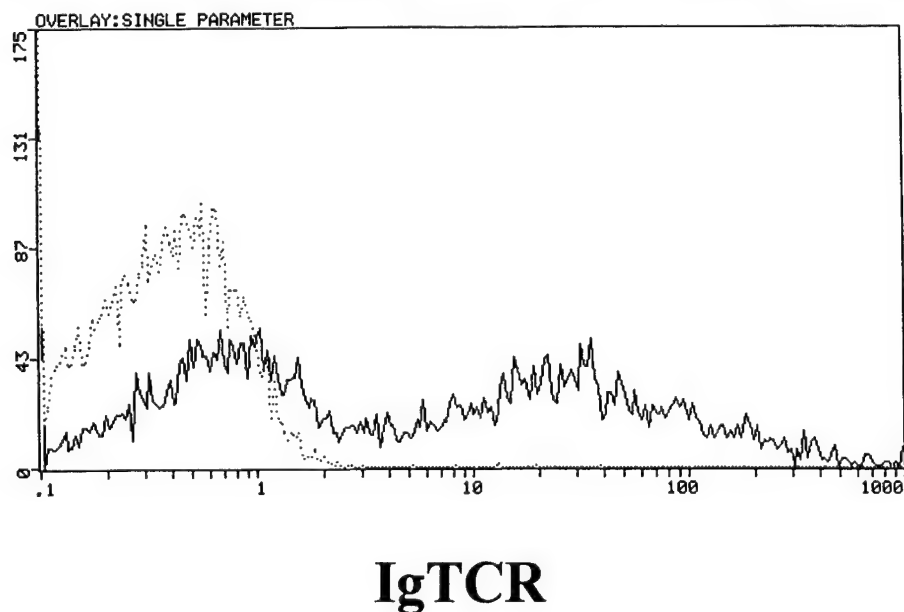

OVERLAY: SINGLE PARAMETER

FIG. 1. Normal human T lymphocytes transduced with the anti-CEA IgTCR vector. OKT3-activated human T cells were infected with the anti-CEA IgTCR vector (see the Materials and Methods section), stained with either PE-labeled WI2 anti-IgTCR antibody (solid lines) or with UPC-negative control antibody (dotted lines), and then evaluated by flow cytometric analysis.

IgTCR

duce T-cell proliferation. Gene-modified T cells were incubated with either immobilized anti-IgTCR antibody or with MIP-CEA tumor cells (Fig. 2). In both cases, ligation of the IgTCR receptor by the target antigen led to increased T-cell death rather than proliferation. In contrast, the same T cells that were cultured in IL-2–containing media alone (not exposed to antigen) continued to proliferate slowly. Although T cells coincubated with tumor cells progressively die, they nonetheless retain potent anti-CEA cytotoxic T-lymphocyte activity for several days [see Beecham et al. (15)].

Stimulation of T Cells Through the Immunoglobulin T-Cell Receptor and CD28 Receptors Induces Rapid and Sustained Proliferation

To determine if costimulating T cells through the IgTCR and CD28 receptors would induce T-cell proliferation, tissue culture plates were coated with anti-CD28 antibody, anti-IgTCR antibody, or both antibodies together. T cells were bound to the plates and cultured in media containing 300 U/mL recombinant IL-2. The number of viable T cells in each treatment was then measured and compared with control cultures that were not stimulated (Fig. 3). Data points are plotted as percentages of
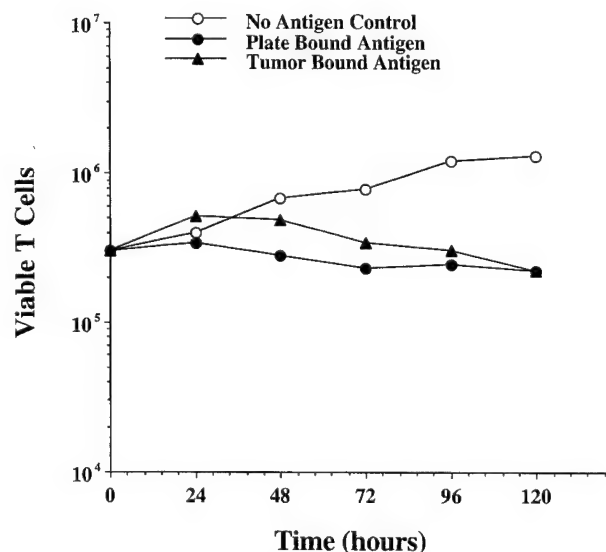
FIG. 3. The addition of signal 2 to signal 1 induces T-cell proliferation. T cells from the experiment described in Figure 1 were incubated in IL-2–containing growth media in tissue culture plates coated with anti-IgTCR antibody alone, with anti-CD28 antibody alone, with anti-IgTCR and anti-CD28 antibodies together, or in IL-2–containing media with no antigenic stimulation as indicated in the legend. Viable T cells were counted (see the Materials and Methods section), normalized to the number of cells present in the untreated controls, and plotted as percentage of controls over time.

control to normalize the cell counts for the slightly different rates of background proliferation in different replicate experiments. T cells stimulated with anti-IgTCR antibody alone (signal 1) proliferated only slightly, whereas cells stimulated with anti-CD28 alone (signal 2) showed no proliferative response. In contrast, T cells stimulated with both anti-IgTCR and anti-CD28 antibodies (signals 1 and 2) began to proliferate rapidly after approximately 5 days and continued to proliferate until the experiment was stopped on day 18. Analogous control experiments on the same cells, but using anti-CD3 antibody, with and without anti-CD28, gave virtually identical results. This shows that the IgTCR and normal TCR signaling pathways function similarly and are both influenced by the presence or absence of CD28 costimulation. It was possible to induce prolonged proliferation with signal 1 alone, but only when plates were coated with antibody concentrations significantly greater than those used in Figure 3. This is in agreement with previous studies on the normal TCRs that have shown that signal 1 alone can induce T-cell proliferation if the signal strength is strong enough (21,22).

Previous studies have indicated that immobilization of antigens on solid surfaces such as plastic culture plates can artificially increase signal strength (21–23). To measure proliferation under more physiologically relevant

FIG. 2. Signal 1 stimulation causes AICD with plate-bound or tumor-bound antigens. T cells (from the experiment described in Figure 1) were incubated in either tissue culture plates coated with anti-IgTCR antibody, in cocultures with MIP-CEA tumor cells, or in regular growth media with no antigenic stimulation as indicated in legend. Viable T cells were counted as described in Materials and Methods, and plotted as a function of time.
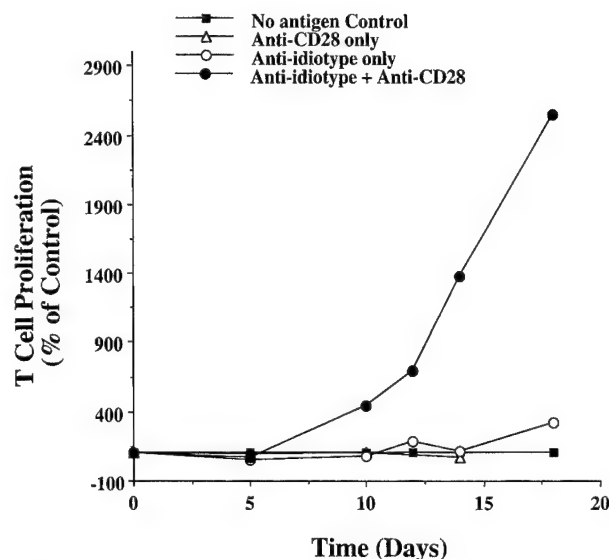
conditions, a second set of experiments was performed in which ligands for signal 1 and signal 2 receptors were displayed on tumor cells. T cells were coincubated with four different tumor cell lines that displayed surface ligands that can cross-link the IgTCR molecule, the CD28 receptor, or both. To generate the appropriate tumor bound ligands, CEA-negative (MIP-101) and CEA-positive (MIP-CEA) colon carcinoma cell lines (24,25) were transduced with a retroviral vector carrying the human B7–1 gene. After selection, appropriate surface expression of the CEA and B7–1 proteins was confirmed by fluorescence-activated cell sorting (Fig. 4).

The growth curves of T cells coincubated with tumor cells expressing CEA alone (signal 1) or CEA and B7.1 (signal 1 and signal 2) are shown in Figure 5A. T cells stimulated with signals 1 and 2 slowly proliferated during the experiment. In contrast, T cells stimulated by signal 1 alone slowly decreased in number. T cells incubated with CEA-negative MIP-101 (no signal) or MIP-101-B7 tumor cells (signal 2 alone) remained relatively unchanged until the plates became overgrown with tumor and were discarded on day 8 (data not shown).

Activation Through the Immunoglobulin T-Cell Receptor and CD28 Receptors Increases Antitumor Efficacy

The differential response of T cells to tumors expressing CEA alone or CEA and B7 was also reflected in the growth or death of the respective tumor lines themselves. Carcinoembryonic antigen–expressing tumor cells are rapidly lysed by anti-CEA IgTCR-modified T cells (15). To maintain antigenic stimulation, these cocultures were periodically fed with fresh MIP-CEA or MIP-CEA-B7 tumor cells. T cells stimulated with signal 1 alone lysed tumor cells for only 7 to 10 days before the continuous addition of tumor cells (MIP-CEA) eventually overwhelmed the lytic capacity of the T cells (Fig. 5B). After this, the tumor cells rapidly proliferate and eventually overgrow the cultures. In contrast, T cells stimulated with signal 1 and 2 (MIP-CEA-B7) were more effective at killing tumor cells (Fig. 5C). In these cocultures, MIP-CEA-B7 tumor cells were added on seven different occasions during the experiment without the outgrowth of tumor. More tumor cell killing in these cultures resulted mainly from T-cell proliferation and the ensuing maintenance of more favorable effector-to-target cell ratios.
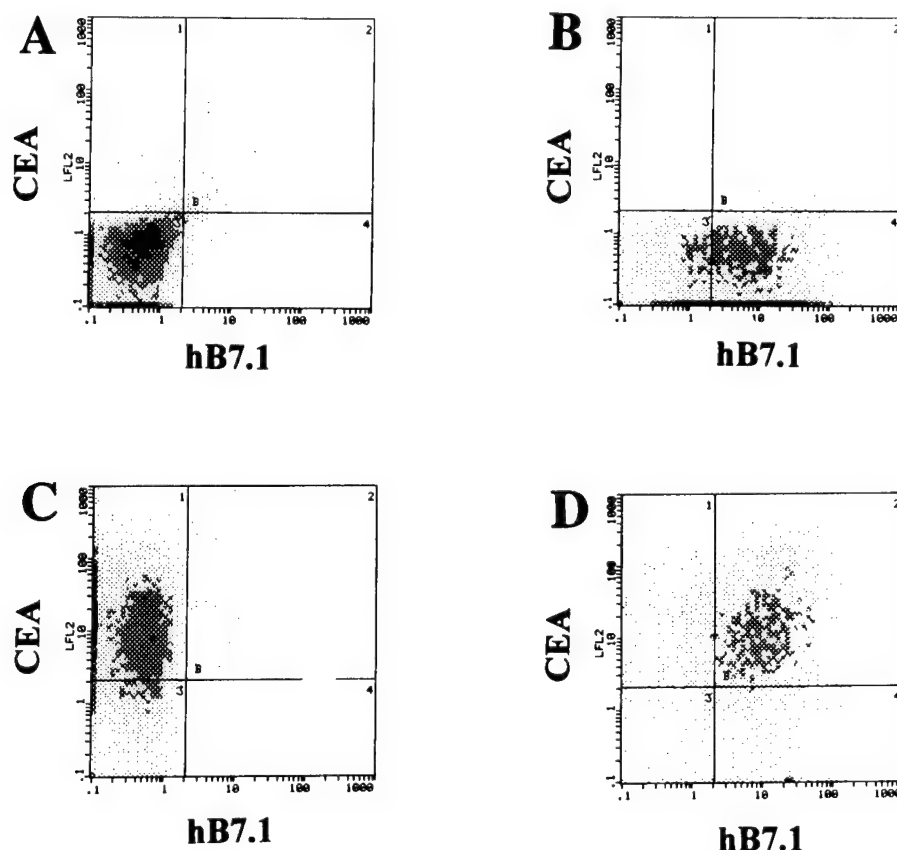

FIG. 4. The human B7–1 gene was introduced into colon carcinoma cell lines. MIP-101 and MIP-CEA tumor cells were transduced with a retroviral vector containing the human B7–1 gene. The parental and transduced tumor cell lines were then stained with PE-labeled hMN14 (CEA expression as shown on the y axis), and fluorescein isothiocyanate–labeled anti-human B7–1 antibody (B7 expression as shown on the x axis), or with the appropriate negative control antibody (HAT-PE and UPC-fluorescein isothiocyanate). Fluorescence-activated cell sorting analysis of cells stained with control antibodies (not shown) were used to set the position of the quadrants in each panel. (A) Parental MIP-101 cells. (B) B7–1 transduced MIP-101 cells. (C) Parental MIP-CEA cells. (D) B7–1 transduced MIP-CEA cells.
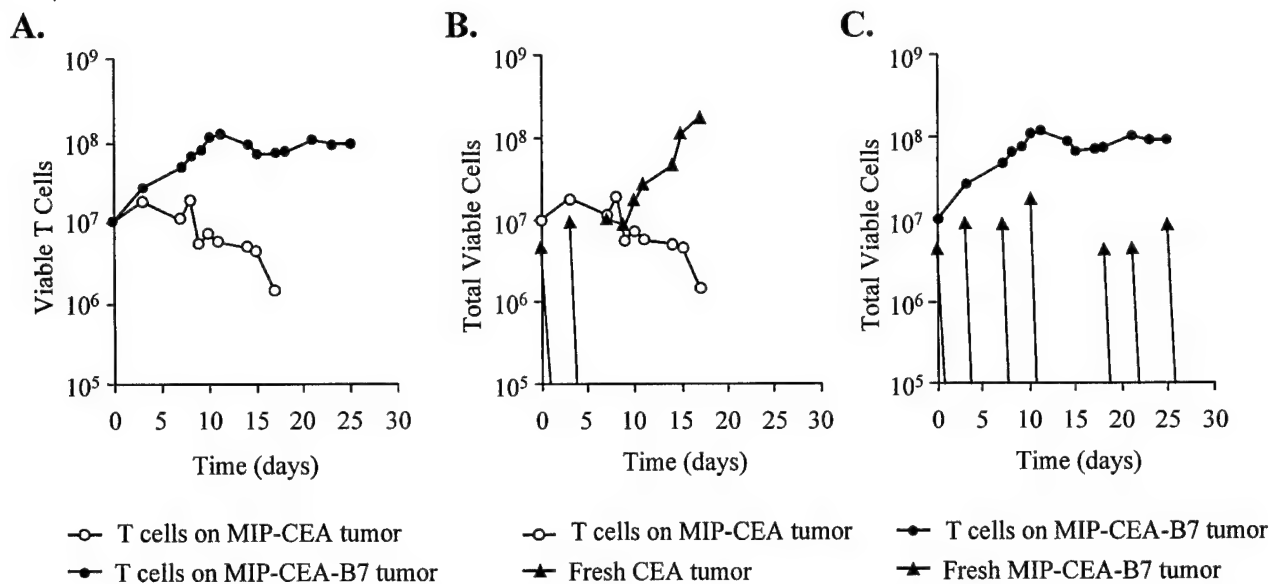
FIG. 5. T cells proliferate in response to tumor cells that display various antigens. T cells from Figure 1 were coincubated with each of the tumor cell lines described in Figure 4 in IL-2–containing growth media. Cocultures were sampled at specific times and counted for both viable T cells and viable tumor cells. (**A**) Counts of viable T cells from cocultures with MIP-CEA or MIP-CEA-B7. Some cultures were terminated before day 27 because of tumor cell overgrowth. Counts of viable tumor cells from cocultures with MIP-CEA (**B**) or MIP-CEA-B7 (**C**). T cell numbers from panel A are separately reproduced in B and C for reference purposes.

Counts of viable tumor cells displaying no ligand (MIP-101) and only signal 2 ligand (MIP-101-B7) showed that both tumor cells lines proliferate rapidly, such that by day 8 the cultures were discarded because of the overgrowth of tumor cells (data not shown).

Signals 1 and 2 Act in Concert to Reduce the Total Amount of Signal Required to Activate Proliferation

Under some circumstances, T cells can be activated to proliferate by signal 1 alone (21–23). Yet, it is also clear that signal 2 plays an important role in controlling T-cell growth (Fig. 3). To define the relative roles of signals 1 and 2, proliferation assays were performed in which the strength of signal 1 was progressively increased in the presence or absence of a constant level of signal 2 (Fig. 6). This was accomplished by assaying T-cell growth in tissue culture plates coated with increasing concentrations of anti-IgTCR antibody alone (signal 1 only), or with anti-IgTCR and anti-CD28 antibodies together (signals 1 and 2). In T cells stimulated with signal 1 alone, no T-cell proliferation was observed until the anti-IgTCR antibody concentration reached very high levels (5 to 20 μg/mL). When T cells were stimulated with signals 1 and 2, proliferation was induced at approximately 500-fold lower levels of signal 1 (0.01 to 0.05 μg/mL). This shows that signal 2 decreases the amount of signal 1 required to trigger proliferation.

In both instances, proliferation was not induced until a specific level of signal 1 strength was reached (the proliferation threshold). One interesting aspect of this analysis is that the range of antigen concentration between no growth and the maximum rate of growth was fairly broad (approximately a 100-fold increase in antigen concentration from minimum to maximum proliferative rates). Furthermore, in both cases, there appeared to be intermediate levels of proliferation within these broad proliferation-induction zones. Thus, signal strength appears to regulate the intensity of the response and its induction. The intensity of the response is finite, however, because once the maximum rate of proliferation was reached, the cells proliferate at about the same rate regardless of whether signal strength was further increased. The maximum rate of proliferation shown in Figure 6 is an approximate 40% increase in cell numbers per day. Even in the presence of adequate levels of signals 1 and 2, there is no net increase in cell number during the first 4 or 5 days (Fig. 3). The values shown in Figure 6 represent average cell growth during a 10-day period, including several days of no net growth. By day 10, maximally stimulated T cells generally doubled once every 18 h (data not shown). Thus, once a proliferative burst is underway, T-cell growth can be explosive.

These results show that T-cell proliferation is triggered by signal 1. This can be accomplished by very high levels of signal 1 alone, or at much lower levels when both signals 1 and 2 are combined. Virtually identical results were obtained in control experiments in which T
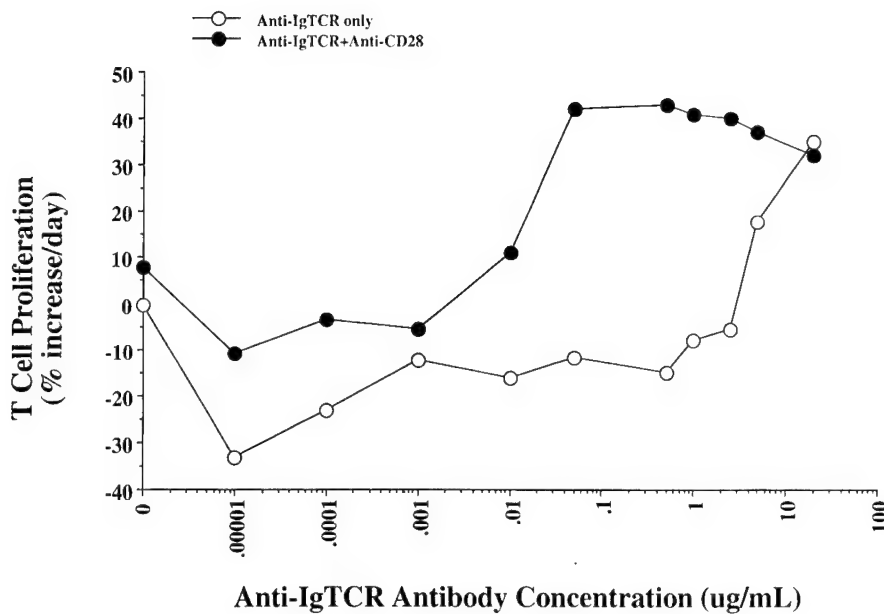
Anti-IgTCR Antibody Concentration (ug/mL)

FIG. 6. T cells proliferated when stimulated with different antigens and at different signal strengths. T cells from the experiment described in Figure 1 were cultured in IL-2–containing growth media in tissue culture plates coated with anti-IgTCR antibody, or with anti-IgTCR and anti-CD28 antibodies together as indicated in the legend. Cultures were assayed for cell proliferation after 10 days of stimulation. Wells were coated with anti-IgTCR antibody at the concentrations shown on the x axis were admixed with either a constant amount of anti-CD28 antibody (10 μg/mL) or with no anti-CD28 antibody. Zero values for the anti-IgTCR antibody concentration shown on the x axis represent either no antibody at all (anti-IgTCR only line) or 10 μg/mL anti-CD28 antibody alone (anti-IgTCR + the anti-CD28 antibody line).

cells were stimulated through the normal TCR, by binding to anti-CD3 antibody alone or in combination with anti-CD28 antibody (data not shown). This indicates that both the normal and chimeric signaling pathways function in similar manners.

Although a specific level of signals 1 and 2 was required to activate proliferation, other indicators of T-cell activation could be induced at much lower levels. In parallel to proliferation, morphologic correlates of activation were also evaluated. When resting T cells are activated, they convert from a single cell suspension of small, round cells to aggregates of large, metabolically active blast cells. Activation-induced changes in morphology were quantified by analyzing forward-scatter

FIG. 7. T-cell morphologic activation is controlled by signal 1. T cells from the experiment described in Figure 6 were analyzed by fluorescence-activated cell sorting for forward-scatter and log side-scatter. (A) Control cells were not exposed to antibody. (B) Cells were stimulated with IgTCR signal 1 only. (C) Cells were stimulated with signals 1 and 2 but did not proliferate (samples were treated with 0.00001 to 0.01 μg/mL). (D) Cells stimulated with signals 1 and 2 proliferated (samples were treated with 0.05 to 5 μg/mL). Dead and dying cells are indicated with arrows, and viable cells are circled.
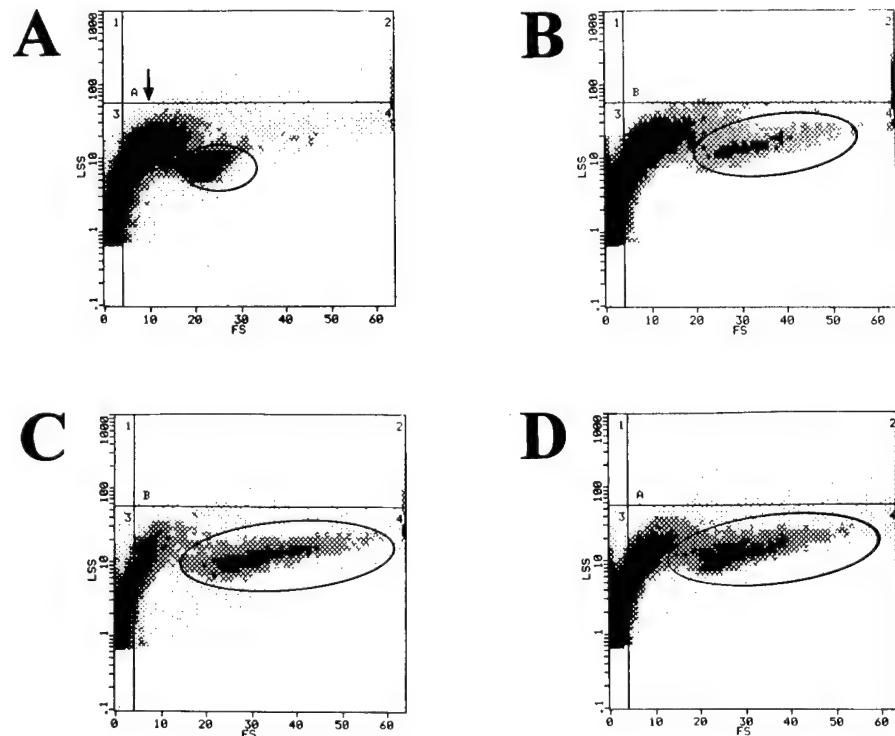
and log side-scatter (FS-LSS) flow cytometric plots of cells stimulated with different signals.

Figure 7A shows the FS-LSS plot for control cells that were not stimulated with either signals 1 or 2. T cells used for these assays (Figs. 6 to 8) were obtained from plateau phase cultures that contain many dead and dying cells (arrow in Fig. 7A). T cells that remain viable in these plateau cultures have a small rounded shape (the circled population in Fig. 7A). When these same cells were stimulated with either signal 1 alone (Fig. 7B) or with signals 1 and 2, (Fig. 7C, nonproliferating cells; Fig. 7D, proliferating cells) they uniformly converted to a blast cell morphology (circled populations) regardless of whether the cells proliferated. By our evaluation, signal 2 had no effect on morphologic change. Thus, different T-cell functions are activated at different levels of signal strength. Some functions (e.g., morphologic activation) are induced by weak or incomplete signals, whereas other functions (e.g., proliferation) require stronger or more complete signals. Similar results were obtained in control experiments in which T cells were stimulated through the normal TCR alone (by anti-CD3 antibody) with and without CD28 costimulation (data not shown), showing that the IgTCR activation pathway mirrors events that occur in the normal activation pathway.

Selective Expansion of Immunoglobulin T-Cell Receptor–Positive Cells With Two Signals

The foregoing experiments suggest an antigen-specific deletion by AICD when T cells receive signal 1 only (Figs. 2 and 5), or an expansion of reactive clones in the presence of costimulation (Figs. 3, 5, and 6). As a test of these principles, we examined changes in the relative proportion of IgTCR-positive cells in the absence or presence of costimulation. The proportions of IgTCR-positive and -negative T cells in unselected cultures were approximately equal (see starting populations of cells in Fig. 8). T cells stimulated through the IgTCR receptor alone underwent a progressive shift to a population of cells that were predominantly IgTCR-negative (Fig. 8B). In contrast, T cells stimulated through both the IgTCR and CD28 receptors shifted to a population that was 100% IgTCR positive (Fig. 8C). As in the experiments described earlier, control experiments were again performed, in which the T cells were stimulated through the normal TCR alone (Fig. 8D) or through the normal TCR and CD28 receptors (Fig. 8E). However, in the current experiments, these control cells reacted differently from those stimulated through the chimeric receptor. T cells stimulated through the normal TCR remained virtually unchanged from the start of the experiments at an ap-
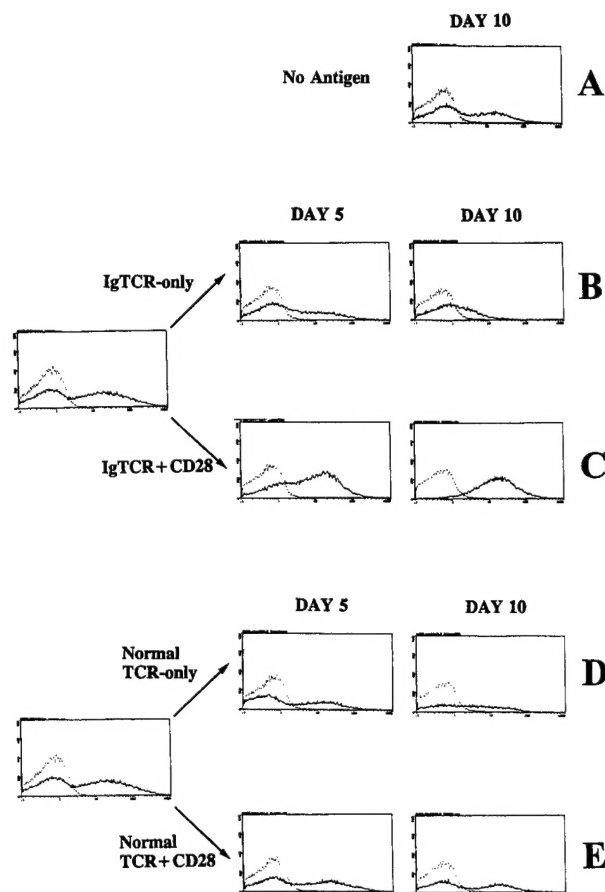

FIG. 8. Complete signals lead to clonal expansion of reactive clones, whereas incomplete signals induce clonal deletion by AICD. T cells were stimulated for 5 and 10 days with signal 1 only, or with signals 1 and 2 as labeled in the figure. Cells were then stained with PE-labeled anti-IgTCR antibody (solid lines) and assayed for IgTCR expression by fluorescence-activated cell sorting. Dotted lines are the negative antibody staining controls.

proximately 50:50 ratio of IgTCR-positive and -negative cells. With the normal TCR, proliferation or AICD affect IgTCR-positive and -negative populations equally, and thus does not change the proportion of positive and negative cells.

DISCUSSION

As one of the three major T-cell responses, antigen-induced proliferation is a critical component of cellular immunity. Antigen-specific T-cell proliferation is particularly important for adoptive immunotherapy, because several studies have shown that ex vivo modified T cells do not circulate well after infusion (28–31). This poor circulation may limit the distribution of modified cells into tumor tissues. In the absence of a proliferative response, the few cells that actually infiltrate tumor tissues may be insufficient to accomplish significant tumor reduction.

Inducing T-Cell Proliferation With Immunoglobulin T-Cell Receptor Molecules Requires CD28 Costimulation

Previous studies (7,8) have reported that IgTCR cross-linking alone is sufficient to activate antigen-specific proliferation. The current study indicates that a productive expansion of IgTCR-modified cells does not generally occur unless the cells also receive CD28 costimulation. Furthermore, T-cell proliferation induced by IgTCR+CD28 activation represents a true expansion in cell number, rather than transient replication that can occur with abortive responses that end in T-cell death. Proliferation could be induced by signal 1 alone (both anti-IgTCR and anti-CD3), but only when high concentrations of cross-linking antibody was immobilized on a solid surface, similar to results obtained by others (21–23). However, these high signal strengths are unlikely to be physiologically relevant. This conclusion is supported by the lack of proliferation when T cells were incubated with MIP-CEA tumor cells. MIP-CEA tumor cells express high levels of cell surface CEA [see Beecham et al. (15)], yet they could not induce proliferation of T cells unless the tumor cells also displayed the B7–1 protein (Fig. 5A).

Costimulation Reduces the Level of Signal Required to Allow Proliferation

The two-signal model holds that costimulation reduces the signaling threshold required to activate T-cell proliferation [see Sperling and Bluestone (17) and Boussiotis et al. (18)]. The current studies confirm that IgTCR-induced proliferation is similarly constrained. However, although signals 1 and 2 are required for T-cell proliferation to occur, costimulation does not lead to fully autonomous cell division. T-cell proliferation still depended on the addition of exogenous IL-2 and rapidly ceased upon IL-2 withdrawal (data not shown). This is consistent with recent reports indicating that, in addition to TCR and CD28, LFA-1 cross-linking is also required to induce the release of effective quantities of IL-2 (32,33). Although costimulation does not fulfill all signal requirements, it does appear to be essential for proliferation at physiologic levels of antigen, because its absence could not be overridden by the presence of exogenous IL-2.

Using an IgTCR molecule against prostate-specific membrane antigen, Gong et al. (34) reported that IL-2 release is induced by coupling CD28 costimulation to IgTCR signals. However, the amount of IL-2 produced in this study was only approximately 3000 pg/mL per 10^6 cells for 24 h (by enzyme-linked immunosorbent assay).

IL-2 release in this study also began to decrease after 48 h. IL-2 in a dose of 3000 pg/mL is equal to approximately 45 IU/mL of IL-2, which is not sufficient, in our experience, to maintain T-cell proliferation (unpublished data). Our studies use different criteria to define IL-2 release. Specifically, we use T-cell proliferation in the absence of exogenous IL-2 as a measure of the biologic consequence of endogenous IL-2 production. This is a more stringent assay readout than enzyme-linked immunosorbent assay because the T cells must synthesize and release enough endogenous IL-2 to maintain autonomous growth. It is likely that CD28 costimulation does induce the release of some endogenous IL-2, particularly because other studies have shown that CD28 costimulation plays a role in regulating the transcription and stability of IL-2 mRNA (35–37). However, our results indicate that the amount of IL2 production induced by IgTCR+CD28 cross-linking is insufficient for maintaining T-cell proliferation.

Incorporating CD28 Costimulation Leads to an Increase in Antitumor Efficacy

Incorporating CD28 signaling into the IgTCR activation pathway increases the overall ability of IgTCR-modified T cells to kill tumor cells. The coculture experiments shown in Figure 5 represent an in vitro model of therapy in which a defined "dose" of T cells are added to tumor cells with and without costimulation. T cells stimulated by signals 1 and 2 lysed a greater total number of tumor cells and maintained their potency for a much longer period of time, whereas T cells stimulated by signal 1 alone were overwhelmed by the rapidly dividing tumor cells. Figure 5 also shows that many more tumor cells were added to the MIP-CEA-B7 cocultures than to the MIP-CEA cultures. When the difference in the number of tumor cells added, and the growth potential of those cells is considered, there is an approximately 200-fold difference in antitumor activity between T cells stimulated with signals 1 and 2 and those stimulated with signal 1 alone. The similar rates of tumor lysis, at fixed effector:target ratios, in both types of coculture have led us to conclude that the increased antitumor efficacy seen with signals 1 and 2 is a result of improved growth and survival of the T cells rather than increased activity of cytotoxic T lymphocytes on a per-cell basis. These results highlight the importance of T-cell proliferation to an effective antitumor response.

Individual T-Cell Effector Functions Have Individual Signaling Requirements

Although T-cell proliferation required a minimum level of signal 1, the presence of signal 2, and exogenous

IL-2, other T-cell functions could be activated without this collaboration. When morphologic criteria are used to define T-cell activation, signal 1 is just as effective as signals 1 and 2 together, and all cells become morphologically activated regardless of whether they proliferate. This indicates that T cell activation proceeds through a stepwise progression in which signal 1 is sufficient to induce some functions while costimulation is required to turn on higher order responses such as proliferation. This makes sense in that TCR recognition (signal 1) must be the first event in the immune response and its stimulation prepares the cell (e.g., upregulation of IL-2R, CD28, LFA-1, metabolic rate, and so forth) to participate in antigen-driven proliferation.

Specific Signals Control T-Cell Entry Into Either an Activation-Induced Cell Death Pathway or a Selective Expansion of Reactive Clones

Analysis of dynamic changes in the proportion of IgTCR-positive cells showed that stimulation with signal 1 alone leads to a progressive and selective deletion of antigen reactive clones (Fig. 8B, IgTCR only). These cultures were not proliferating and the conversion of the population to predominately IgTCR-negative cells resulted from the selective death of IgTCR-positive cells. In contrast, T cells stimulated with signals 1 and 2 progressively shifted from a 50:50 mixture of IgTCR-negative and IgTCR-positive cells to a population that was 100% IgTCR-positive (Fig. 8B, IgTCR+CD28) through the selective outgrowth of T cells that expressed higher levels of the receptor. Subclones that express quantitatively higher levels of receptor can generate stronger signals than subclones that express low levels of receptor simply because there are more receptors that can be cross-linked. This stronger signaling potentially provides high expressing cells with a selective advantage. When costimulation is present, this translates into a growth advantage for high expressors. When costimulation is absent, AICD results in the deletion of high expressors.

These dynamic changes are similar to events that occur during normal immune responses. Previous studies have shown that the repertoire of antigen-responsive subclones will evolve during in vivo T-cell expansions. This evolution involves dynamic changes in the proportions of different subclones such that the response becomes focused on specific epitopes (38–40). In some responses, this repertoire focusing is caused by the selective expansion of subclones with TCRαβ chains that have higher binding affinities (41). In contrast, the evolution of high expressing IgTCR subclones in Figure 8 does not arise

because IgTCR molecules vary in their affinity for antigen. All IgTCR subclones express the same chimeric receptor with the same receptor affinity. Rather, IgTCR subclones differ in the total number of receptors expressed per cell, and high levels of receptor on specific subclones leads to stronger binding, greater signal strength, and more rapid proliferation in those clones. By this mechanism, high expressing subclones evolve to become the predominant subtypes. Thus, the end result of increased TCR cross-linking is analogous between the two systems, despite the qualitative differences between them.

In conclusion, the current study shows that CD28 costimulation is required to induce proliferation of IgTCR-stimulated human T cells. Previous reports that show ^{3}H-thymidine incorporation (7,8) after IgTCR cross-linking most likely measured transient DNA replication that can occur during abortive responses (27). Proliferative responses that occur with IgTCR and CD28 costimulation are prolonged expansions that more closely resemble what occurs during a productive native immune response. The current study also shows that IgTCR-induced activation proceeds in a stepwise manner in which weaker signals will induce morphologic activation but not proliferation. We previously showed that cytolytic functions are fully induced by IgTCR signals alone and do not require either CD28 or exogenous IL-2 (15). Thus, the first step of T-cell activation appears to involve a group of responses that include morphologic, metabolic, and cytolytic functions. Productive proliferation, on the other hand, appears to be one of the final steps of activation and requires not only signals 1 and 2 and sufficient quantities of IL-2 but also a level of signal 1 that is greater than a specific threshold. This threshold also appears to be the point at which the T cell commits to either an AICD pathway (absence of signal 2) or to a pathway of clonal expansion and affinity selection (with signal 2). Finally, the integration of CD28 costimulation into the IgTCR-activation pathway may increase antitumor responses in clinical applications of IgTCR-based immunotherapies. We are currently testing the ability of IgTCR and IgCD28 dual chimera vectors to direct antigen-specific cytolysis and proliferation. Because IL-2 is not released in this setting, a third level of activation may be required for a more complete self-sustaining antitumor response that does not require the addition of exogenous IL-2.

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